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NL 98 51 00453

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REC'D 01 OCT 1999

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Patentanmeldung Nr. Patent application No. Demande de brevet n°

98204215.2

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**Blatt 2 der Bescheinigung  
Sheet 2 of the certificate  
Page 2 de l'attestation**

Anmeldung Nr.:  
Application no.:  
Demande n°: 98204215.2

Anmeldetag:  
Date of filing: 14/12/98  
Date de dépôt:

Anmelder:  
Applicant(s):  
Demandeur(s):  
CENTRUM VOOR PLANTENVEREDELINGS- EN REPRODUKTIEONDERZOEK (CPRO-DLO)  
6708 PB Wageningen  
NETHERLANDS

Bezeichnung der Erfindung:  
Title of the invention:  
Titre de l'invention:  
Process to collect metabolites from modified nectar by insects

In Anspruch genommene Priorität(en) / Priority(ies) claimed / Priorité(s) revendiquée(s)

Staat:  
State:  
Pays:

Tag:  
Date:  
Date:

Aktenzeichen:  
File no.  
Numéro de dépôt:

Internationale Patentklassifikation:  
International Patent classification:  
Classification internationale des brevets:  
/

Am Anmeldetag benannte Vertragsstaaten:  
Contracting states designated at date of filing: AT/BE/CH/CY/DE/DK/ES/FI/FR/GB/GR/IE/IT/LI/LU/MC/NL/PT/SE  
Etats contractants désignés lors du dépôt:

Bemerkungen:  
Remarks:  
Remarques:

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14 12 1998

Process to collect metabolites from modified nectar by insects.

#### Field of the invention

5 The present invention relates to isolated, purified DNA sequences which can act as promoters in eukaryotic cells. More specifically, the present invention is related to such DNA sequences which act as promoters to express genes in nectaries of plants. The present invention also relates to chimerical gene constructs comprising a structural or a  
10 synthetic gene under the control of a promoter that effects expression of said genes in nectaries. This invention also relates to a process for producing metabolites in honey by allowing insects, preferably bees, to collect and process nectar from plants that excrete said metabolites in nectar  
15 or other exudates. Further, this invention relates to plant cells, plants or derivatives therefrom, that express the said chimerical gene.

#### Background of the invention

20 Nectaries are nectar secreting organs or tissues that can be located inside (floral) or outside (extrafloral) the flower. The main component of nectar is sugar, the variation between nectars of flowers from different species mainly  
25 being the concentration and ratio of glucose, fructose and sucrose (Baker and Baker, 1982). In addition, depending on the plant species, varying amounts of polysaccharides, lipids, organic acids, volatiles, minerals, phosphates, alkaloids, amino acids and proteins have been detected

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(Baker and Baker, 1982). Being a specialised sink organ, the nectaries are supplied with sucrose by phloem unloading (Davis et al., 1985, Hagitzer and Fahn, 1992).

- 5 The mechanisms of sugar accumulation and nectar secretion have been described for several plant species (Fahn et al., 1979). Sugar transport to the nectaries is achieved by active transport mechanisms and/or osmotic and chemical gradients. In the nectaries of many plants sucrose is  
10 converted to glucose and fructose, resulting in a hexose dominant nectar. Part of the hexoses are converted to starch, which is hydrolysed prior to anthesis and nectar secretion. Cell to cell transport of nectar in the nectary parenchyma tissue is mainly symplastic, as demonstrated by  
15 the presence of many plasmodesmata between these cells (Fahn et al., 1979). Nectar is secreted from secretory cells via the cell membrane (eccrine secretion) or via the Golgi and endoplasmatic reticulum vesicles (granulocrine secretion). Research on the molecular regulation of nectary  
20 development and nectary biochemistry has not been reported.

- The main function of floral nectar is to reward pollinating insects. Insects collect nectar to meet their short-term energy requirements. Colony-living honeybees process large  
25 quantities of nectar into honey, which is stored in honeycombs of the beehive and is used as food supply during the winter period. Within the bee colony different classes of worker bees cooperate in the honey production process. Foraging bees collect pollen and nectar from the flowers  
30 and bring it to the hive. On returning to the hive they give most of it up to household bees. Pollen is used as a protein source, especially to feed the brood. Adult nurse and worker bees use little protein, their capacity to digest proteins being very low (Crailsheim et al., 1993).  
35 Honey processing takes place by repeated swallowing and bringing up of the nectar from the honey stomach. In the first process 15% of the water content is lost. This semi-

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processed nectar is temporarily stored in a honeycomb cell and taken out later for further processing. The final process includes filtering the honey to discard small particles like pollen grains. Sugar metabolising enzymes (invertase, amylase) are added and the honey is concentrated to an average water content of 20%. Most nectars and honeys only contain traces of protein ( $<0.2\%$ ). However, *Calluna vulgaris* (heather) honey can contain up to 1.8% protein, giving it thixotropic properties (Butler, 1962). It is known that bees add enzymes like invertase to nectar during the honey processing. Therefore, the probability that proteases are also added is very low. Protein digestion does not take place in the honey stomach but in the intestine of the honeybee. However, the ability of adult worker bees to digest proteins is very low, their main requirement being energy which they obtain from nectar. Until now, it was not established which proteins are present in heather honey and whether these originate from floral heather nectar or are added to honey by honeybees.

In the present invention it was established that heather honey contains two unique proteins that originate from floral nectar of heather. Based on these results a production system for proteins in nectar and honey was established.

It is an object of the present invention to show that recombinant proteins can be secreted in nectar of transgenic plants, that this nectar is collected by honeybees and that the bees process this nectar into honey that contains the unaltered protein in a concentrated form.

#### Definitions

**Honey:** A substance that contains approximately 80% sugar and varying amounts of other components and that is produced by insects, preferably bees, that collect and process

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nectar from floral or extrafloral nectaries, from honeydew, other plant exudates or artificial sugar solutions.

5 MADS box gene: a gene coding for a transcription factor having a region of 56 amino acids which is homologous to a similar region in the Arabidopsis AGAMOUS protein and Antirrhinum DEFICIENS protein. This region is called the 'MADS box'. At least 50% of the amino acids in this region should be identical to the amino acid composition in the  
10 MADS boxes of AGAMOUS and/or DEFICIENS.

Nectary: secretory organ or secretory tissue of plants, located in the flowers (floral nectaries) or outside the flower (extrafloral nectaries) that excrete nectar.

15 Nectar: sugar containing fluid that is secreted by nectaries. Nectar can also contain substances like minerals, amino acids, proteins, organic acids, volatiles, alkaloids etc.

20 Recombinant protein: the gene product of a recombinant DNA molecule.

25 Recombinant DNA molecule: A DNA molecule in which sequences which are not naturally contiguous have been placed next to each other by in vitro manipulations.

Promoter: The DNA region, usually upstream to the coding sequence of a gene, which binds RNA polymerase and directs  
30 the enzyme to the correct transcriptional start site.

#### Summary of the invention

35 The production of recombinant proteins for pharmaceutical purposes is a growing market. Until now, mainly bacterial and yeast systems have been used for bulk production of proteins. Recently animal production systems have also been



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developed. With the availability of efficient transformation techniques for plants, procedures to use plants for the production of proteins are now in progress. In plants, the recombinant proteins are targeted to sink organs like tubers and seeds. A serious draw-back of these production methods is that the recombinant protein can only be obtained after extended, and therefore expensive, purification steps.

The present invention provides a method to produce metabolites, preferably recombinant proteins in honey, which is manufactured by insects, preferably honeybees, that collect floral nectar of transgenic plants. Harvesting of honey is very simple and purification of the protein is very straight forward and requires no advanced purification steps. To give an estimation of the protein yield in a crop like rapeseed, we suggest an average protein production of 2% in honey, as has been found in honey of heather. If one hectare of rapeseed yields 100-500 kilo honey in one season, a yield of 2 to 10 kilo protein can be obtained. In addition, the present invention provides a method to collect metabolites from honey that is derived from non-transgenic plants that secrete these metabolites in nectar. An example are secondary metabolites like acetylcholine, a diterpene compound, that is excreted in nectar of *Rhododendron arboreum* and *Rhododendron barbatum* and of *Piptanthus nepalensis* (Martini et al., 1990).

This invention provides a gene from petunia, *NEC1*, that is highly expressed in the nectaries of petunia and weakly expressed in the stamens. It also provides another gene from petunia, *FBP15*, that encodes a MADS box protein and which is specifically expressed in the nectaries of petunia. Further, it provides the isolated DNA sequences of the promoters of the *NEC1* and the *FBP15* genes. Furthermore, this invention provides an isolated DNA sequence expressed in nectaries encoding a signal peptide that is translation-

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ally fused to a germin-like protein (Lane et al., 1993, Dumas et al., 1995), having the function to target the mature germin-like protein to nectar of heather (*Calluna vulgaris*). This invention gives proof that protein-containing sugar solution is collected by honeybees to produce honey that has a higher protein content than the sugar solution itself, the protein having undergone no qualitative alterations. This invention also proves that a recombinant protein can be produced in nectar of transgenic plants and that this protein is present in honey produced by honeybees that collected this nectar.

Accordingly, this invention provides an isolated DNA sequence which encodes a protein indicated NEC1 and having the amino acid sequence given in SEQ ID NO:1 of the sequence listing hereafter or homologs of NEC1. A homolog of NEC1 is predominantly expressed in nectaries and/or has at least 60% homology with the amino acid sequence given in SEQ ID NO:1. Further this invention provides an isolated DNA sequence which encodes a protein indicated FBP15 and having the amino acid sequence given in SEQ ID NO:2 of the sequence listing hereafter or a homolog of FBP15. A homolog of FBP15 is specifically expressed in nectaries and belonging to the MADS box family. Furthermore, a homolog is also a gene sequence that has at least 80% homology within the MADS box region and a 60% overall homology with the amino acid sequence given in SEQ ID NO:2. Further this invention provides the characterisation and the isolation of a DNA sequence which encodes a signal peptide indicated "CVSP" (*Calluna vulgaris* signal peptide), wherein the information contained in the DNA sequence permits, upon translational fusion with a DNA sequence encoding a protein that is expressed in nectaries, targeting of the protein to nectar.

The DNA sequences of the invention can also be characterised in that they comprise the NEC1 gene and the FBP15 gene having the nucleotide sequences given in SEQ ID NO:4 and

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SEQ ID NO:5 respectively, or a functionally homologous gene or an essentially identical nucleotide sequence or part thereof or derivatives thereof which are derived from said sequences by insertion, deletion or substitution of one or more nucleotides, said derived nucleotide sequences being obtainable by hybridisation with the nucleotide sequences given in SEQ ID NO:4 and 5 respectively.

Furthermore, the DNA sequences of the invention can also be characterised in that they comprise signal sequence CVSP having the nucleotide sequence given in SEQ ID NO:6, or an essentially identical nucleotide sequence or part thereof or derivatives thereof which are derived from said sequences by insertion, deletion or substitution of one or more nucleotides.

Further, this invention provides an isolated DNA sequence from the promoter region upstream of a nectary-specific expressed sequence, which nectary-specific expressed sequence encodes a protein comprising the amino acid sequence given in SEQ ID NO:1, or a homologous protein.

Furthermore, this invention provides an isolated DNA sequence from the promoter region upstream of a nectary-specific expressed sequence, which nectary-specific expressed sequence encodes a protein comprising the amino acid sequence given in SEQ ID NO:2, or a homologous protein.

In a further aspect, the invention provides a protein encoded by any of the above defined DNA sequences. Further, the invention provides processes of producing transgenic plants exhibiting excretion of recombinant proteins in nectar, the expression of the chimerical genes and the targeting of the recombinant proteins being under the control of promoter sequences and a signal sequence as described in this invention. Still further, the invention provides processes of producing transgenic plants that produce recombinant proteins in nectar, the expression of

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the chimerical genes being under the control of promoter regions upstream of other genes that are expressed in nectaries. Still further, the invention provides processes of producing transgenic plants that produce recombinant proteins in nectar, the expression of these proteins being under the control of any signal peptide that affects targeting of a protein in nectar.

Also, the invention provides recombinant double stranded DNA molecules comprising expression cassettes to be used in the above process. Further, the invention provides transgenic bacteria, transgenic plants producing recombinant proteins in nectar, and also plant cells, tissue culture, plant parts or progeny plants derived from said transgenic plants. Finally, the invention provides a process to produce recombinant gene products in honey, produced by bees that collect nectar from transgenic plants and process this nectar into honey.

20

#### Brief description of the figures:

25

Figure 1 shows a polyacrylamide gel with PCR products after Differential Display mRNA amplification. PCR reactions were performed with the oligo-dT primer T12MG in combination with 5 different random primers Ap11-AP15 on cDNA samples of pistils without nectaries (two independent samples), nectaries (two independent samples), leaves and a mixture of sepals (s), petals (p) and stamens (a). Bold arrow depicts the cloned fragment DD18.

35 Figure 2 is the DNA sequence of the Differential Display RT-PCR clone DD18a. The primers prat 122 and prat 119 that were used for 5' RACE PCR reactions are underlined.

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Figure 3 is the DNA sequence of clone RC8, obtained by RACE PCR with gene specific primers prat 122 and prat 119 (Fig. 2) in combination with adapter primers. Primer prat 129 (underlined) is used in the next step together with primer  
5 prat 122 to amplify the coding region of the *NEC1* cDNA.

Figure 4 is the full length sequence of *NEC1* cDNA. The translation start (ATG) and translation stop (TAA) are depicted bold.

10

Figure 5 shows the expression of *NEC1* (A) and *FBP15* (B) in wild type petunia plants (line W115) as determined by Northern blot analysis. Blot A contains total RNA, while blot B is enriched for mRNA. The tissues are indicated as:  
15 1= leaf, 2= sepal, 3= petal, 4= stamen, 5= pistil, 6= nectary. For blot A the HindIII/EcoRI fragment of pDD18a was used as a probe. For blot B the full length cDNA of *FBP15* was used as a probe.

20 Figure 6 Expression of *NEC1* by in situ localisation of *NEC1* transcripts (A) and activity of the *NEC1* promoter in the nectaries (B) and the stamen (C) as shown by GUS expression driven by the *NEC1* promoter. The GUS assay used for the stamens was incubated overnight without modificati-  
25 ons to prevent diffusion (example 8). The GUS assay for the nectaries was incubated for 5 hrs, using an assay mixture to prevent diffusion (example 8). For in situ localisation longitudinal sections of flowers of *Petunia hybrida* were hybridised with digoxigenin-labeled antisense *NEC1* RNA

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Figure 7 is the DNA sequence from the promoter region upstream of a sequence encoding the *NEC1* protein. Underlined is the translation start of *NEC1* cDNA.

35 Figure 8 depicts a schematic presentation of the T-DNA region between the borders of the binary vector pBNEP1, containing the *NEC1* promoter (Figure 7), the GUS reporter

- 10 -

gene and the nos terminator in pBINPLUS. This vector was used to generate transgenic plants to study the expression of the NEC1 promoter.

- 5 Figure 9 shows the SDS-PAGE separation of proteins that are present in commercial honey samples from different flowers. M= marker, lane 1: wattle bark, lane 2: flower mixture, lane 3: heather, lane 4: clover, lane 5: rapeseed.
- 10 Figure 10 shows the SDS-PAGE separation of proteins that are present in commercial honey samples of rapeseed (RH2x, RH10x) and heather (HH2x, HH10x) and of nectar samples of rapeseed (RN2x, RN10x) and heather (HN2x, HN10x). M= molecular weight marker. Two (2x) or ten (10x) fold dilutions were used.
- 15

Figure 11 shows the SDS-PAGE separation of proteins present in dilutions of the sugar/BSA feeding solution (A) and of honey from bees that had collected the sugar/BSA solution (B). The dilutions of the sugar/BSA and honey/BSA solution was the same for both gels: 1= 15x, 2= 30x, 3= 60x, 4= 75x, 5= 75x, 6= 90x, 7= 105x, 8= 120x, 9= 135x. M= marker

20

Figure 12 shows the sequence homology of the N-terminal protein sequence of CVH29, a unique protein present in heather honey and nectar, with a germin-like protein GER1 from a gene bank homology search (BLAST).

25

Figure 13 shows the deduced DNA sequence of the N-terminal protein sequence of CVH29. The degenerated primers prat 176 and prat 177 are underlined (A). The DNA sequence of the PCR product obtained with prat 176 and prat 177 performed on genomic DNA of heather is shown in B. The gene-specific primers prat 207 and prat 206 used to perform 5'RACE PCR reactions on cDNA from heather flowers are underlined.

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Figure 14 shows the DNA sequence of four independent clones obtained by 5'RACE PCR with prat 207 and prat 206 on cDNA of heather flowers. The ATG translation start of the putative signal sequence is boxed. The end of the putative  
5 signal sequence and the start of the mature protein are indicated by arrows.

Figure 15 is the sequence of the synthetically produced DNA molecule encoding the signal sequence CVSP (boxed) with  
10 linkers.

Figure 16 is the schematic representation of the plasmid pCV1. Not all restriction sites are indicated.

15 Figure 17 is the schematic representation of the plasmid pCV2. Not all restriction sites are indicated.

Figure 18 is the schematic representation of the plasmid pCV3. Not all restriction sites are indicated.

20 Figure 19 is the DNA sequence of the full length cDNA of FBP15. The translation start (ATG) and translation stop (TAA) are boxed. The MAD-box and K-box region are underlined.

#### 25 Detailed description of the invention

This invention provides processes of producing transgenic  
30 plants that produce recombinant proteins in nectaries and nectar that is collected by foraging honeybees. This invention gives evidence that honeybees process protein containing nectar into honey that contains the unaltered protein in a concentrated form. Subsequently, the desired  
35 protein can be purified from the honey.

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To express recombinant proteins in nectaries of transgenic plants, a translational fusion of an isolated DNA sequence from a promoter region upstream of a sequence encoding a protein that is expressed in nectaries with a sequence  
5 encoding the recombinant protein has to be carried out. Preferably, the isolated DNA sequence from a promoter region is upstream of a sequence that is specifically or highly expressed in nectaries.

10 The invention relates to a DNA sequence isolated from *Petunia hybrida* that encodes a protein indicated NEC1 or a homologous protein or part thereof. A homologous protein has at least 65% homology with the amino acid sequence given in SEQ ID NO:1. The cDNA sequence of the NEC1 gene is  
15 given in Fig. 4 and in SEQ ID NO:4. The deduced amino acid sequence of the NEC1 gene is given in SEQ ID NO:1. The NEC1 gene shows strong expression in the nectaries and in a very localised region of the anther filaments of *Petunia hybrida*. The deduced amino acid sequence of NEC1 predicts a  
20 membrane bound protein. The precise function of the gene has not been elucidated yet, but considering the phenotype of transgenic plants that ectopically express NEC1 in the leaves, a role in sugar metabolism of NEC1 is apparent.

25 The present invention also relates to homologous DNA sequences that can be isolated from other organisms, preferably plants, using standard methods and the already known DNA sequence of the NEC1 gene. More precisely, it is also possible to use DNA sequences which have a high degree  
30 of homology to the DNA sequence of the NEC1 gene, but which are not completely identical, in the process according to the invention. The use of sequences having homologies between 85 and 100 % is to be preferred. DNA sequences can also be used which result from the sequence shown in SEQ ID  
35 NO:4 by insertion, deletion or substitution of one or more nucleotides. This includes naturally occurring variations or variations introduced through targeted mutagenesis or



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recombination. The DNA sequence shown in SEQ ID NO:4 can also be produced by using DNA synthesis techniques.

The invention also relates to a DNA sequence isolated from  
5 *Petunia hybrida* that encodes a MADS box protein indicated  
FBP15 or a homologous protein or part thereof. The cDNA  
sequence of FBP15 is given in SEQ ID NO:5. FBP15 shows  
exclusively expression in the nectaries of *Petunia hybrida*.  
The function of FBP15 is unknown.

10 The present invention also relates to homologous DNA  
sequences that can be isolated from other organisms,  
preferably plants, using standard methods and the already  
known DNA sequence of FBP15. More precisely, it is also  
possible to use DNA sequences which have a high degree of  
15 homology to the DNA sequence of FBP15, but which are not  
completely identical, in the process according to the  
invention. The use of sequences having homologies between  
85 and 100 % is to be preferred. DNA sequences can also be  
used which result from the sequence shown in SEQ ID NO:5 by  
20 insertion, deletion or substitution of one or more nucleo-  
tides. This includes naturally occurring variations or  
variations introduced through targeted mutagenesis or  
recombination. The DNA sequence shown in SEQ ID NO:5 can  
also be produced by using current DNA synthesis techniques.

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Further, this invention provides an isolated DNA sequence  
from the promoter region upstream of a nectary-specific  
expressed sequence, which nectary-specific expressed  
30 sequence encodes a protein comprising the amino acid  
sequence given in SEQ ID NO:1, or a homologous protein that  
is expressed in nectaries. Furthermore, this invention  
provides an isolated DNA sequence from the promoter region  
upstream of an isolated DNA sequence from the promoter  
35 region upstream of a nectary-specific expressed sequence,  
which nectary specific sequence encodes a protein compri-

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sing the amino acid sequence given in SEQ ID NO:2, or a homologous protein that is expressed in nectaries.

5 More specifically this invention provides an isolated DNA sequence from the promoter region upstream of a nectary-specific expressed sequence, which nectary-specific expressed sequence has:

- a) a nucleotide sequence given in SEQ ID NO:4, or
  - b) a nucleotide sequence obtainable by hybridisation with
- 10 the nucleotide sequence of (a) or with a fragment of (a).

15 In a more specific embodiment this invention provides an isolated DNA sequence from the promoter region upstream of a nectary-specific expressed sequence, obtained from a plant of *Petunia hybrida*, the sequence consisting essentially of the sequence given in SEQ ID NO:7, or a functional fragment thereof having promoter activity.

20 In a further aspect, the invention provides an isolated DNA sequence from the promoter region upstream of a nectary-specific expressed sequence, which nectary-specific expressed sequence has:

- a) a nucleotide sequence given in SEQ ID NO:5, or
  - b) a nucleotide sequence obtainable by hybridisation with
- 25 the nucleotide sequence of (a) or with a fragment of (a).

30 In a more specific embodiment this invention provides an isolated DNA sequence from the promoter region upstream of a nectary-specific expressed sequence, obtained from a plant of *Petunia hybrida*, the sequence consisting essentially of the sequence given in SEQ ID NO:8, or a functional fragment thereof having promoter activity.

35 Further, this invention provides an isolated DNA sequence comprising the coding region for a signal peptide, wherein the information contained in the DNA sequence permits, upon translational fusion with a DNA sequence encoding a protein

- 15 -

that is expressed in nectaries, targeting of the protein to nectar. More specifically, the DNA sequence comprises the nucleotide sequence given in SEQ ID NO:6 obtained from a plant of *Calluna vulgaris*, or a nucleotide sequence obtainable by hybridisation with the nucleotide sequence given in SEQ ID NO:6. The use of sequences having homologies between 95 and 100 % is to be preferred. DNA sequences can also be used which result from the sequence shown in SEQ ID NO:6 by insertion, deletion or substitution of one or more nucleotides. This includes naturally occurring variations or variations introduced through targeted mutagenesis or recombination. The DNA sequence shown in SEQ ID NO:6 can also be produced by using DNA synthesis techniques. The signal peptide CVSP was isolated from nectar of *Calluna vulgaris* flowers and from honey processed by honeybees that collected the nectar. The function of CVSP in heather nectaries is to target the germin-like protein to nectar. The DNA sequence CVSP can also be used to target other proteins to nectar in plant species.

A subject of the present invention is the use of DNA sequences for producing recombinant proteins in nectar of plants, wherein the protein is produced in nectaries and targeted to nectar, and wherein expression in nectaries is achieved by using a DNA sequence consisting of the promoter region upstream of a DNA sequence that is expressed in nectaries, and wherein secretion in nectar is achieved by using a DNA sequence that encodes a signal sequence that targets the recombinant protein to nectar. In a further aspect the present invention relates to processes wherein a recombinant protein is expressed in other plant tissues than the nectaries and wherein the biochemical composition of nectar is changed as a consequence of the recombinant gene expression. The present invention also relates to processes wherein a recombinant protein is expressed in nectaries of a transgenic plant, wherein the biochemical composition of nectar or the nectar secretion is changed as

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a consequence of this protein expression. In particular, it relates to processes where the recombinant protein is an enzyme that interferes with the sugar metabolism in nectaries.

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The production of a recombinant protein in nectaries and nectar is achieved by integrating into the genome of the plants a recombinant double-stranded DNA molecule comprising an expression cassette having the following constituents and expressing it:

10

- i) a promoter functional in nectaries of plants,
- ii) a DNA sequence encoding a protein which is fused to the promoter,
- iii) a DNA sequence encoding a signal peptide that targets the recombinant protein to nectar, which is translationally fused to the DNA sequence encoding the recombinant protein, and optionally
- iv) a signal sequence functional in plants for the transcription termination and polyadenylation of an RNA molecule.

15

20

Such DNA molecules are also subject of the invention. The present invention provides an example of such a DNA molecule that contains the described expression cassettes in the form of plasmid pCV3 (Fig. 18), which comprises the promoter region of the *NEC1* gene from petunia, the signal sequence CVSP from heather, the coding region of the reporter gene *GUS* and the NOS terminator. In principle, any promoter that is active in the nectaries of plants can be used as promoter. The promoter is to ensure that the chosen gene is expressed in nectaries. Also, in principle, any signal sequence that targets the expressed protein to nectar can be used as a signal sequence. The signal sequence is to ensure that the protein is excreted in nectar.

Furthermore, any sequence that encodes a recombinant protein in nectaries can be used in the present invention. Preferably, the subject of this invention relates to DNA

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sequences that encode proteins to be used for pharmaceutical purposes. It is also possible to use the invention to produce proteins for other purposes, e.g. enzymes for biotests or antioxidants for food additives. Furthermore, it is possible to use the invention to produce metabolites in nectar that attract predators of pest insects or that kill or repel pest insects. In another aspect it is possible to use the invention to produce metabolites in nectar that modify the attractiveness of the plant for pollinating insects or improve the health of pollinating insects.

It is also possible to use DNA sequences that encode proteins that modify the nectar composition or the sink strength of nectaries. This means that the recombinant protein interferes with metabolic pathways in the nectaries, resulting in changed levels of compounds that are already present in nectar, or the formation of new compounds in nectar.

In addition, the present invention also relates to expression cassettes that contain the above mentioned DNA sequences, except for a signal sequence. The recombinant protein is then only expressed in the nectaries, but not targeted to the nectar. Consequently, the expression of the recombinant protein in the nectaries can still affect nectar composition.

In a further aspect, the present invention also relates to expression cassettes that contain DNA sequences coding for a protein that is expressed in other tissues than the nectaries. The expression of the recombinant protein affects changes in the biochemical composition of nectar or in nectar secretion.

Finally, the present invention also relates to non-transgenic plants that produce metabolites in nectar that can be harvested and purified from honey that is produced by honeybees that collect this nectar. Examples for these metabolites are alkaloids, terpenes, amino acids, proteins, pigments and volatiles.

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A preferred embodiment of the process discussed above provides that the expression cassette is transformed to a plant species that produces nectar. Preferably, the recombinant protein is produced in nectar of plants that are visited by honeybees that collect the nectar. Honeybees collect floral as well as extrafloral nectar. The present invention relates to plants that produce recombinant proteins in floral or extrafloral nectar. In addition, the present invention also relates to plants that produce recombinant proteins in other plant organs, said plant organs producing an exudate that is collected by insects, preferably bees, and processed into honey. A particularly preferred embodiment of the present invention are plants that can be grown under controlled conditions. Controlled conditions are greenhouses or field facilities where transgenic plants can be grown according to the safety rules that are required. Preferably, the controlled conditions are such that bee colonies that perform normal foraging behaviour can be maintained in the same compartment during the flowering period. Preferred plants originate from the Brassicaceae family, in particular *Brassica napus*.

#### Examples

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##### Example 1:

##### Cloning of *NEC1*

The *NEC1* cDNA was isolated using the mRNA Differential Display system (Genhunter Corporation, Brookline USA). The isolation of total RNA from nectaries, sepals, petals, stamens and pistils from open flowers and from young leaves of *Petunia hybrida* was done according to Verwoerd et al. (1989). Two independent RNA isolations were performed on nectaries as well as on pistils. A DNase treatment was carried out on each RNA sample, using the RNA MessageClean™ Kit (Genhunter Corporation Brookline USA, cat. No. M601). A

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reverse transcription reaction was carried out on 0.1  $\mu$ g RNA of each sample, using the oligo-dT primer T12MG from the Genhunter Kit. Following the protocol, PCR reactions were carried out using the arbitrary primers AP11-AP15 in combination with primer T12MG from the Kit. The PCR products were loaded on a sequencing gel and after electrophoresis the gel was blotted on 3M paper, dried and exposed to X-ray film (Figure 1). Two adjacent nectary-specific bands were cut out from the blot and the DNA was purified according to the manual. Reamplification of the fragment was carried out using the oligo-dT primer T12MG and the arbitrary primer AP15. After electrophoresis, the PCR product was extracted from the agarose gel by freezing the isolated fragment in liquid nitrogen, followed by centrifugation. DNA was precipitated by adding 1/10 volume 1% HAc, 0.1M MgCl<sub>2</sub>, and 2.5 volume of 96% ethanol to the supernatant. The pellet was dissolved in 10  $\mu$ l TE buffer. The fragment, now called DD18a, was cloned into a PMOSBlue T-vector (RPN 1719, Amersham Little Chalfont UK) giving the vector pDD18a.

The nucleotide sequence of this 3' cDNA clone was determined by the dideoxynucleotide chain termination method (ABI PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit, P/N 402078, Perkin Elmer) and is shown in Figure 2. The DNA fragment has a length of 460 nucleotides. The missing 5' part of the cDNA was isolated using the Marathon™ cDNA Amplification Kit of Clontech (catalog K1802-1) and following the procedure as described in the manual. Briefly, Poly A+ RNA was isolated from nectaries of *Petunia hybrida* flowers. After double stranded cDNA synthesis, adapters were ligated and a 5' RACE reaction was carried out using the adapter primer AP1 supplied in the kit and a gene-specific primer prat 122. The nucleotide sequence of prat 122 is: 5'-gtgggaaggctatgctacaagc-3' (Figure 2). The PCR product was diluted 10x and 1  $\mu$ l was used in a second 5' RACE reaction with the nested adapter primer supplied by the kit (AP2) and the nested gene-specific primer prat 119

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(Figure 2). The nucleotide sequence of prat 119 is: 5'-ccttctccatggactgcaatgcg-'3. After gel electrophoreses a fragment of  $\pm 850$  bp was obtained that hybridised with clone DD18a. The fragment, now called RC8, was extracted from the gel, purified and cloned into a PMOSBlue T-vector as described above. The sequence is shown in Figure 3. The combined (overlapping) sequences of clones DD18a and RC8 are shown in Figure 4, comprising the full length cDNA of a gene called *NEC1* hereafter. The *NEC1* clone has a length of 1205 nucleotides and encodes for a polypeptide of 265 amino acid residues. Based on the deduced amino acid sequence, high homology was found with a cDNA that is associated with *Rhizobium*-induced nodule development in the legume *Medicago trunculata* (MtN3, gene bank number: gn1/PID/e274341). The percentages of identity and similarity are 47% and 72% respectively. Analysis of the predicted protein, using the CAOS/CAMM programme (Protein analysis 1991, Genetics Computer Group inc., Wisconsin USA), shows that the putative protein structure resembles membrane proteins, having six evenly spaced hydrophobic loops that traverse the cell membrane. In addition, a signal sequence is predicted at the N-terminus, while the C-terminus is highly hydrophilic. Highest homology with MtN3 is found in the N-terminal signal sequence, the first two membrane-spanning loops and the last two membrane-spanning loops. The C-terminal hydrophilic part shows the lowest homology (28% identity, 30% similarity). The function of *NEC1* has not yet been determined.

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**Example 2:****Cloning of *FBP15***

Petunia MADS box cDNA clones were isolated from a cDNA library made from nectaries of *Petunia hybrida* flowers. The cDNA library was constructed using the lambda ZAP cloning vector (Stratagene, La Jolla USA, catalog nr. 200400-



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200402). The library was screened under low stringency hybridisation conditions with a mixed probe comprising the MADS box regions of Floral binding protein gene *FBP2*, *FBP6* and *pMADS3* (Angenent et al., 1993, 1994, Tsuchimoto 1993).

5 The hybridizing phage plaques were purified using standard techniques. Using the in vivo excision method, *E. coli* clones which contain a double-stranded Bluescript SK-plasmid with the cDNA insertion between the *EcoRI* and *XhoI* cleavage site of the polylinker were generated. Cross-  
10 hybridisation of the purified clones revealed 3 independent clones that did not cross hybridise with previously isolated *FBP* cDNA's and which were designated *FBP15*, *FBP16* and *FBP17*. The nucleotide sequence of *FBP15* was determined by the dideoxynucleotide-mediated chain termination method and  
15 is depicted in SEQ ID NO:5. The *FBP15* cDNA clone has a length of 1157 nucleotides and encodes a peptide of 222 amino acid residues. All characteristics of a MADS box protein are present in *FBP15*: a N-terminal located MADS box region which shows a high degree of similarity with other  
20 MADS box proteins, and a K-box in the middle of the protein with an alpha helical structure. *FBP15* is most similar to the tobacco MADS box protein *NAG1*, which is an Agamous homolog and expressed in whorl 3 and 4 (Huang et al., 1996, Mizukami et al., 1996).

### 25 Example 3:

#### Expression of *FBP15*

Expression of *FBP15* was determined by standard Northern  
30 blot hybridisation experiments. A DNA fragment comprising the complete cDNA of *FBP15* was used as a probe. High stringency hybridisation and washing conditions were used. Using 10 µg of total RNA from various petunia tissues, expression of *FBP15* was only detectable in nectaries. Using  
35 10 µg of mRNA from various tissues, prepared by using the kit and protocol of the Quickprep Micro mRNA Purification

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Kit (Pharmacia Biotech), expression of *FBP15* was only detectable in nectaries as shown in Figure 5B. The expression in the ovary and nectaries was determined by in situ hybridisation using a DIG labelled antisense RNA probe corresponding to the full length cDNA of *FBP15*. In vitro antisense RNA transcripts were made using T7 RNA polymerase. A standard protocol for in situ hybridisation was used as described by Canās et al., 1994. A hybridizing signal was observed evenly strong in all cells of the nectary tissue.

#### Example 4:

##### 15 Expression of *NEC1*

The RNA expression of *NEC1* was determined by standard Northern blot hybridisation experiments. A DNA fragment comprising the complete sequence of the Differential Display clone DD18 (Figure 2) was used as a probe. Using 10 µg of total RNA from various petunia tissues, strong expression of *NEC1* was detectable in nectaries and weak expression in anthers. No expression was detectable in other floral organs, in leaves or in roots (Figure 5A).

25 The expression in the ovary and nectaries was determined by in situ hybridisation using a DIG labelled antisense RNA probe corresponding to the nucleotides 79 to 1036 of *NEC1* cDNA, comprising the coding region and part of the 3' untranslated region. A clone containing this sequence was obtained by PCR on adapter-ligated cDNA, using two gene-specific primers prat 122 and prat 129 (Figure 4). The nucleotide sequence of prat 122 is: 5'-gtgggaaggctatgctacagc-3'', comprising the nucleotides 1015 to 1036 of the *NEC1* cDNA. The nucleotide sequence of prat 129 is: 5'-gggatccatgagcgaattacatgctg-3', comprising the nucleotides 79 to 100 of the *NEC1* cDNA. The gene-specific region of the primers is underlined. The primer contains an extra

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BamHI and NcoI site at the 5' end. A PCR fragment of 958 nucleotides was obtained and cloned into a PMOSBlue vector. The fragment was subcloned in a vector containing the T7 promoter and in vitro antisense RNA transcripts were made using T7 RNA polymerase. A standard protocol for in situ hybridisation was used as described by Canās et al., 1994. Strong hybridizing signals were observed in the outer cell layers of the nectaries (Figure 6A)

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#### Example 5:

#### Isolation of *NEC1* promoter fragment

15 The promoter fragment of *NEC1* was cloned using the genome walker protocol (PT3042-1) and kit as provided by Clontech Laboratories. Briefly, genomic DNA from *Petunia hybrida* was digested with 5 different blunt cutting restriction enzymes. GenomeWalker adapters were ligated and PCR reactions were carried out on each GenomeWalker "library" with a gene specific, reversed primer prat 148 and the adapter primer from the kit (AP1). The nucleotide sequence of prat 148 is: 5'-ccaagaaggccaaatatgaaagac-3' comprising the nucleotides 105 to 128 of the *NEC1* cDNA (Figure 4). PCR products were subjected to a second round of PCR, using the nested adapter primer AP2 and the nested gene specific, reversed primer prat 149. The nucleotide sequence of prat 149 is: 5'-aagtcacacgacgtaattgcgcc-3', comprising the nucleotides 81 to 104 of the *NEC1* cDNA. From the second PCR a 2 kb fragment was isolated from the *StuI* library, which was cloned in the PMOSBlue T-vector, yielding the construct pMAS-10. Figure 7 (SEQ ID NO:7) shows the DNA sequence of the *NEC1* promoter in the construct pMAS-10, including the translation start of *NEC1* cDNA.

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## Example 6:

## Construction of NEC1 promoter-GUS

A PCR reaction was performed on pMA5-10 (example 5), using  
5 the forward vector primer U19 of pMOSBlue and the gene-  
specific primer prat 169. The nucleotide sequence of prat  
169 is:  
5'-cgctgcagcgccatggttttttttagtgaagcccc-3'. The gene-speci-  
fic region is underlined. The primer contains an NcoI and  
10 BglII restriction site at the 3' end. The PCR product was  
digested with KpnI and NcoI and ligated into a pBluescript-  
derived vector (pMO4) that contains the NTM19 promoter  
(Custers et al., 1997), the reporter gene *GUS* and the nos  
terminator. The KpnI/NcoI NTM19 promoter fragment was  
15 replaced, resulting in a NEC1-promoter/*GUS* translational  
fusion. The resulting plasmid pNEP1 was digested with SmaI  
to release the NEC1 promoter/*GUS*/nos fragment and this  
fragment was ligated into a derivative of the binary  
plasmid pBIN (Bevan, 1984) yielding the binary plasmid  
20 pBNEP1 (Figure 8). pBNEP1 was introduced into *Agrobacterium*  
*tumefaciens* strain LBA4404 or C58pMP90 by electroporation.  
Plasmid DNA from the *Agrobacterium* transformants was  
isolated and the structure of the binary vector was veri-  
fied by restriction analysis and PCR.

25

## Example 7:

Generation of transgenic *Petunia* plants

*Agrobacterium* strain LBA4404 transformants were used to  
30 transform *Petunia hybrida* using leaf discs as described by  
Horsch et al. (1985). After shoot and root induction on  
kanamycin selection media, plants were transferred to soil  
in the greenhouse.

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Example 8:

Histochemical GUS assay

5 Different plant parts of Kanamycin-resistant plants transformed with the pBNEP1 construct were analysed for the distribution of  $\beta$ -glucuronidase activity (GUS) using the method described by (Jefferson et al., 1987). In transgenic plants with high expression levels diffusion of reaction products to other tissues was observed. To avoid this spreading a modified GUS assay was used. Briefly, tissues were pre-treated with 90% cold acetone at -20°C for 1 h, then rinsed three times 20' with 100 mM phosphate buffer containing 1 mM potassium ferricyanide. After this treatment the standard GUS assay was performed with the modification that ferricyanide was excluded from the reaction mixture.

Example 9:

Results histochemical GUS assay

20 In very young flowers (<1,4 cm) no blue staining was observed, in flowers of 2-4 cm weak blue staining of the nectaries was observed. In flowers of (4-6 cm) strong blue staining was observed in the nectaries (figure 6B) and in a very restricted region of the upper part of the anther filaments (Figure 6C). GUS expression was highest in the outer cell layers of the nectary parenchyma. In cross sections of the anther filaments GUS expression was observed in all cells except in the xylem of the inner vascular bundle.

Example 10:

Protein analysis of heather honey and nectar

35 Samples of pure heather honey, together with samples of rapeseed, clover, wattle bark and lavender honey were diluted, dialysed and loaded on a 12% SDS page gel (Laemmli,

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1970). All honey samples showed several identical high molecular weight protein bands. Heather honey contained 2 unique protein bands of 29 and 50 kDa (Figure 9). The proteins were named CVH29 and CVH50 (CVH stands for *Calluna vulgaris* honey). To determine the origin of the proteins, nectar and honey samples of rapeseed and heather were prepared and loaded on a 12% SDS page gel. The high molecular weight protein bands of around 70 kDa that are present in all honey samples were not observed in rapeseed or heather nectar (Figure 10). These proteins are added by honeybees during honey processing. Proteins CVH29 and CVH50 are present in heather honey and heather nectar, but not in nectar of rapeseed. Therefore, it was concluded that CVH29 and CVH50 are secreted in nectar of heather and can be recovered from honey derived from this nectar. The protein concentration in the heather honey we tested was around 0.5%.

#### Example 11:

#### 20 N-terminal sequence analysis of CVH29 and CVH50

Honey samples were loaded on an SDS PAGE gel and after electrophoreses the gel was blotted on a PVDF membrane. After staining the CVH29 and CVH50 bands were cut out from the blot and N-terminal sequencing was performed on both proteins. The N-terminal sequence of CVH50 is: SVLDFCVADPS-LPDGPAGYSCTEPSTVTSQDF. The N-terminal sequence of CVH29 is: SVLDFCVADPSLPDGPAGYSCKEPAKVTVDDEFVHGLGTA. A gene bank homology search (BLAST) showed high amino acid sequence homology (63%) with germin-like proteins isolated from Arabidopsis (Figure 12).

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## Example 12:

Identification signal sequence of CVH29

Because the germin-like protein CVH29 is excreted in  
5 heather nectar it was expected that part of the cDNA  
encodes a signal sequence. Based on the N-terminal amino  
acid sequence, degenerated primers were designed. The  
sequence of the forward primer prat 176 is: 5'-gayt tyt-  
gygtngcngaycc-3' (y= c or t, n= c, t, a or g). The sequence  
10 of the reversed primer prat 177 is: ccrtgraanacraartcrtc  
(r= g or a). A PCR reaction performed on genomic DNA of  
heather yielded a 99 bp DNA fragment. The fragment was  
sequenced and two reversed, gene-specific 5' primers were  
designed to clone the 5' cDNA by "Marathon cDNA racing"  
15 using the kit and protocol of Clontech laboratories (proto-  
col PT1115-1, Clontech Palo Alto USA). The sequence of  
gene-specific primer prat 207 that was used is: 5'-  
ggtgacttttagagggctccttgc-3', the sequence of gene-specific  
nested primer prat 206 is:  
20 5'-gctccttgcaggagtagcctgc-3' (Figure 13). RNA was isolated  
from open flowers of heather and mRNA was prepared using  
the Pharmacia quickprep micro mRNA kit. After cDNA synthe-  
sis and adapter ligation a PCR reaction was performed,  
using the adapter primer AP1 and the gene-specific primer  
25 prat 207. The PCR product was used for a second PCR, using  
adapter primer AP2 and the nested gene-specific primer prat  
206. A single fragment of around 300 nucleotides was  
obtained and cloned in a PMOSBlue T-vector. Four clones  
were sequenced. Figure 14 shows that three clones were  
30 identical and one clone had two different nucleotides in  
the untranslated 5' region. A putative signal sequence of  
17 amino acids was identified between the ATG start codon  
and the first codon of the mature protein CVH29 that was  
identical in all four clones. The nucleotide sequence of  
35 the putative signal sequence (SEQ ID NO:6) is:  
5'-atgtttcttccaattctcttcaccatttcctcctctctctcctcccatgct-  
3'.

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## Example 13:

Construction of an expression cassette for excretion of proteins in nectar

5 To clone the *NEC1* promoter into a PMOSBlue vector a PCR reaction was carried out on pMA5-10 (example 5) using the forward primer prat 247 and the reversed primer prat 248 (Fig. 7). Prat 247 contains an extra *Pst*I restriction site. The *Nde*I restriction site of prat 248 coincides with the  
10 ATG translation start of *NEC1*. The nucleotide sequence of prat 247 is: 5'-ggctgcaggagtgttctttgataaatg-3', the nucleotide sequence of prat 248 is: 5'-cgccatattgttttttatggaagcccc-3'. Gene-specific regions are underlined. A 1,8 kb promoter fragment was obtained and  
15 cloned into a pMOSBlue vector, yielding the plasmid pNECP.

A DNA molecule encoding the signal sequence CVSP as depicted in SEQ ID NO:6 was produced by synthesis and subsequent annealing of two oligo molecules prat 245 and prat 246. The  
20 sequence of prat 245 is: 5'-tatgttccttccaattcttttcactatttctcttcttttcttcttctcatgcttctgttcttgatttc'3, the sequence of prat 246 is: 5'-gatccgaaatcaagaacagaagcatgagaagaagagaaaagaa-gagaaatagtgaaaagaattggaaggaaca'3. The region encoding the signal sequence CVSP is underlined. To ensure correct  
25 cleavage of the signal peptide, the linkers were extended with the coding region for the first five amino acids of the mature germin-like protein (Fig. 13). The codon usage of the signal peptide sequence was optimised for Arabidopsis. By addition of a *Bam*HI restriction site at the 3' end,  
30 2 extra amino acids were linked in frame to the mature protein. The resulting DNA molecule is shown in Figure 15. The fragment was ligated into a *Nde*I/*Bam*HI cut PMOSBlue vector, yielding the plasmid pCVSP.

35 pNECP was digested with *Nde*I and *Pst*I to release the *NEC1* promoter fragment which was cloned into the *Pst*I/*Nde*I cut



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pCVSP, yielding the plasmid pCV1. A schematic representation of pCV1 is given in Figure 16.

5 A, 250 bp long fragment containing the NOS terminator sequence (NOST) was obtained by PCR, using the forward primer prat 251 and the reversed primer prat 252 on DNA of pRAP 33, which is a pUC 19 derived plasmid. Prat 251 adds a SacI and XhoI site, prat 252 adds a SmaI and EcoRI site. The sequence of prat 251 is: 5'-gggagctcgagtcggttcaaa-  
10 catttggcaataaaq-3'. The sequence of prat 252 is: 5'-cgaatt-  
cccggggatctagtaacataagatgacac-3'. The NOST-specific regions are underlined. The PCR product was cloned into pCR-Script™ Amp SK(+) Cloning Kit (Catalog 21188-21190, Stratagene La Jolla USA), yielding the plasmid pCR-NOST. pCR-NOST was  
15 digested with SacI and EcoRI and the resulting fragment was cloned into the pUC 19 (ClonTech), derived plasmid pUCAP yielding the plasmid pCVNOS.

20 The plasmid pGUSN358 was purchased from Clontech (catalog 6030-1) containing the reporter gene GUS in pUC 119, modified to destroy the N-linked glycosylation site within the 1.814 Kb GUS coding sequence. A PCR reaction was carried out with gene-specific primers prat 249 and prat  
25 250, yielding a fragment that contains the GUS gene coding region and a BamHI restriction site at the 5' end and a SacI restriction site at the 3' end. The sequence of prat 249 is: 5'-ccggatccatgattacgtcctgtagaaacc-3'. The sequence of prat  
30 250 is: 5'-gggagctcccaccgaagctatag-3'. The GUS specific regions are underlined. Subsequently, the PCR fragment was digested with BamHI and SacI and ligated into the BamHI/SacI cut plasmid pCVNOS, yielding the plasmid pCV2. A schematic representation of pCV2 is given in Figure 17.

35 pCV1 is digested with PstI and BamHI and the resulting fragment is cloned into the PstI/BamHI cut plasmid pCV2, yielding the plasmid pCV3. A schematic representation of pCV3 is given in Figure 18. pCV3 is digested with AscI and

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SmaI and the resulting fragment is cloned into a derivative of the binary plasmid pBIN, yielding the binary plasmid pBCV3. pBCV3 was transferred from *Escherichia coli* to the *Agrobacterium tumefaciens* strain LBA4404 and C58pMP90 by electroporation. The transformed *Agrobacterium* strain was used to transform *Arabidopsis* and *petunia*.

**Example 14:**

## Protein production in nectar

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Using the GUS reporter gene, GUS activity in nectar of transgenic plants was measured according to the method as described by Jefferson et al., (1987). Briefly, the assay was carried out by measuring the amount of methyl umbelliferone (MU) produced by GUS fluorometrically by emission of light of 455 nm. The absolute emission was corrected for artificial quenching using an internal standard of 1nM MU (Angenent et al., 1993).

20

**Example 15:**

## Feeding experiments with honeybees

In September 1996 a beehive located outside was supplied with a 25% sucrose solution supplemented with 2% BSA (bovine serum albumin). After 3 weeks the bees had consumed 15 litters of the feeding solution and honey was harvested from the hive. Although the flowering season had mostly past, bees still foraged on flowers to collect nectar outside. Therefore, the honey produced during this period is derived from a mixture of the feeding solution and nectar from flowers. An SDS page protein gel was loaded with dialysed honey samples and sugar/BSA solutions. Figure 11 shows that the protein band of BSA was present in all the samples tested and no qualitative changes were observed in the honey samples compared to the sugar/BSA solutions. The BSA concentration in honey was 1.5 times higher than in the feeding samples, demonstrating that protein is concen-

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trated in honey. Honeybees that foraged on the sugar/BSA solution did not show any aberrant behaviour and the colony developed normally.

5 Example 16:

Process of honey production from transgenic plants

10 Twohundred and fifty transgenic plants that each produce recombinant protein in nectar were grown in a greenhouse of 25 square meters. The facilities were adjusted according to the safety rules according to European law, including safety measures to prevent in- or outflow of insects. A  
15 beehive adjusted for small populations, containing around 200 worker honeybees and a queen, was placed in the greenhouse when the plants were flowering. When a queen is present, she will start laying eggs and larvae will come out. The presence of brood stimulates the bees to collect nectar and process it into honey. After 2-3 weeks bees  
20 processed the nectar into honey and stored in sealed cells of the honeycomb. Under the described conditions the amount of honey that can be harvested is 250-1000 grams.

Example 17:

25 Ablation of nectaries

By introducing the highly sensitive Rnase BARNASE in plant cells, under the control of a tissue-specific promoter, cell ablation can be achieved in very specific tissues or  
30 organs. Ablation of nectaries can be applied to decrease the attractiveness of plants for pest insects that forage on the nectar that is secreted by nectaries. In addition, plants without nectaries can be obtained that are more resistant to bacterial and fungal infections. An example is  
35 given for the ablation of nectary tissue by expressing bacterial BARNASE in nectaries, using the NEC1 promoter.

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Plasmid DNA of pNEP1 (example 6) was digested with KpnI and NcoI to release the 1800 bp NEC1 promoter fragment. The purified promoter fragment was ligated into a pWP90 derived vector, upstream of the BARNASE-BARSTAR bacterial operon construct (Hartley, 1988). The construct contains a 35SCaMV terminator of polyA signal cauliflower mosaic virus terminator sequence downstream of the BARNASE-BARSTAR operon. The resulting plasmid pWP126 was digested with KpnI/ XhoI to release the NEC1-promoter/BARNASE-BARSTAR/CamVpolyA fragment and this fragment was ligated into a pBIN-derived vector pBIN Plus. The recombinant vector was transferred via *Agrobacterium tumefaciens* (LBA4404) to petunia variety W115. Transgenic petunia plants were selected with flowers without nectaries or underdeveloped nectaries.

Many promoters are less specific than can be concluded based on promoter/GUS expression is concluded. Because the bacterial BARNASE is highly cytotoxic at very low concentrations it can be preferred to protect other plant tissues by expression of a ribonuclease inhibitor gene under the control of a weak, constitutive promoter (e.g. NOS promoter) or a tissue-specific promoter that is not active in the tissues where cell ablation is to be achieved (Mariani et al., 1992, Beals et al., 1997).

30

Example 18:  
Ectopic nectary development

MADS box genes regulate floral meristem and floral organ identity. Ectopic expression of MADS box genes can change the developmental fate of floral organs or cells. Transgenic petunia plants ectopically expressing FBP11, an ovule-

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specific MADS box gene, develop ovule-like structures on sepals and petals (Colombo et al., 1995). *FBP15* is a nectary-specific MADS box gene, involved in the molecular regulation of nectary development. In petunia nectaries develop at the base of the carpel. Ectopic expression of *FBP15* in petunia may result in the development of nectaries on other organs of the flower or on vegetative parts of the plant. An example is given of a gene construct that, when transformed to a plant, results in ectopic expression of *FBP15*.

*FBP15* was amplified using a 5' primer that hybridises with *FBP15* sequences just upstream of the ATG translation start site and a 3' primer that hybridises with *FBP15* sequences just downstream the translation stop site. The 5' primer contains a *NcoI* recognition site, the 3' primer contains a *BamHI* recognition site. After the sequence was confirmed, the amplified *FBP15* fragment was inserted as a *BamHI/NcoI* fragment into the binary vector pCPO31. This binary vector was derived from pPCV708, as described by Florack et al. (1994), and contains three expression cassettes with a multiple cloning site between the left and right T-DNA borders. The cDNA was cloned in sense orientation between a modified *CaMV* 35S promoter and the nopaline synthase terminator sequence. The chimerical gene construct was transferred via *Agrobacterium* GV3101 to petunia variety W115, using the transformation method as described in example 7. Transgenic petunia plants were selected that show ectopic nectary development.

#### Example 19:

Modification of sugar composition and nectar secretion

Although sugar content of nectar from different petunia W115 flowers shows some variation, the ratio between hexoses and sucrose is very stable. Down-regulation or up-regulation of genes involved in the establishment of the

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ratio between hexoses and sucrose in nectar will therefore modify nectar composition. An example is given for anti-sense expression of a petunia-derived invertase gene.

5 PCR primers were designed that hybridise with the cDNA of an invertase gene cloned from *Solanum tuberosum*. The 5' primer 5'-AAGGACTTTAGAGAGACCCGACCACTGCTGG-3' and the 3' primer 5'-AAATGTCTTTGATGCATAATATTTCCCATAATC-3' were used for a PCR reaction on genomic DNA of petunia to yield a  
10 fragment of around 420 bp. The fragment was sequenced and cloned into a pMOSBlue vector to used as a probe to screen a petunia nectary-specific cDNA library. Hybridizing phage plaques were purified and cDNAs were retrieved by in vivo excision as described in example 2. The expression of the  
15 cDNA's was determined by Northern blotting as described in example 3 and the sequence of a nectary-specific invertase was determined as described in example 2. The invertase gene was amplified using a 5' primer that hybridises with sequences just upstream of the ATG translation start site  
20 and a 3' primer that hybridises with sequences just downstream of the translation stop site. Extra restriction enzyme recognition sites were generated to allow cloning of the cDNA in sense (overexpression) or antisense direction into the binary vector pCPO31 as described in example 18.  
25 The chimerical gene constructs are transferred via *Agrobacterium* GV3101 to petunia variety W115, using the transformation method as described in example 7. Transgenic petunia plants were selected that exhibit modified sugar composition in nectar.

30

#### Example 20

#### Modification of plant development

35 A DNA which is the *NEC1* gene or a homologous gene is introduced into a plant cell, the said DNA being induced by promoter elements controlling the expression of the introduced DNA in such a way that transcription produces

- 35 -

sense RNA. Plants were regenerated from the transgenic cells as described in example 7. Plants that ectopically express the *NEC1* gene exhibited modified leaf morphology and modified sugar composition. Furthermore, plants that ectopically express the *NEC1* gene showed a delay in flowering time.

## 10 REFERENCES:

Angenent G.C., Franken J., Busscher M., Colombo L. and van Tunen A.J., 1993. Petal and stamen formation in petunia is regulated by the homeotic gene *fbp1*. *Plant J.* 4, 101-112.

Angenent G.C., Franken J., Busscher M., Weiss D. and van Tunen A.J., 1994. Co-suppression of the petunia homeotic gene *fbp2* affects the identity of the generative meristem. *Plant J.* 5, 33-44.

Baker H.G. and Baker I., 1982. Floral nectar constituents in relation to pollinator type. In: *Handbook of experimental pollination biology* (ED. by CE Jones and RJ Little), pp 117-141. Scientific and Academic Edition, Van Nostrand Reinhold, New York.

Beals T.P. and Goldberg R.B. (1997). A novel cell ablation strategy blocks tobacco anther dehiscence. *The Plant Cell* 9, 1527-1545.

Bevan M. (1984). Binary *Agrobacterium* vectors for plant transformation. *Nucl. Acids Res.* 12, 8711-8721

Butler C.G. (1962). *The world of the honeybee*. London: Collins.

- 36 -

- Canās L.A., Busscher M., Angenent G.C., Beltran J-P and van Tunen A.J. (1994). Nuclear localisation of the petunia MADS box gene protein FBP1. *Plant J.* 6, 597-604.
- 5 Colombo L., Franken J., Koetje E., van Went J., Dons H.J.M., Angenent G.C. and van Tunen A.J. (1995). The petunia MADS Box gene FBP11 determines ovule identity. *The Plant Cell* 7, 1859-1868.
- 10 Crailsheim k., Hrassnigg N., Lorenz W and Lass A. Protein consumption and distribution in a honeybee colony (*Apis mellifera carnica* Pollm). *Apidologie* 24 (5), 510-511.
- 15 Custers J.B.M., Oldenhof, Schrauwen J.A.M., Cordewener J.H.G., Wullems G.J. and Lookeren Campagne M.M. (1997). Analysis of microspore-specific promoters in transgenic tobacco. *Plant molecular biology* 35, 689-699.
- 20 Davis A.R., Peterson R.L. and Shuel R.W. (1986). Anatomy and vasculature of the floral nectaries of *Brassica napus* (Brassicaceae). *Can. J. Bot.* vol. 64, 2508-2516.
- 25 Dumas B., Freyssinet G. and Pallett E. (1995). Tissue-specific expression of germin-like oxalate oxidase during development and fungal infection of barley seedlings. *Plant Physiol.* 107: 1091-1096.
- Fahn A. (1979). Ultrastructure of nectaries in relation to nectar secretion. *Amer. J. Bot.* 66(8), 977-985.
- 30 Florack D.E.A, Dirkse W.G., Visser B., F. Heidekamp and Stiekema W.J. (1994). Expression of biologically active hordothionins in tobacco: Effects of pre- and pro-sequences at the amino and carboxyl termini of the hordothionin precursor on mature protein expression and sorting. *Plant Mol. Biol.* 24. 83-96.
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- Hartley R.W. (1988). Barnase and Barstar: Expression of its cloned inhibitor permits expression of a cloned ribonuclease. *J. Mol. Biol.* 202, 913-915
- 5 Horsch R.B., Fry J.E., Hofman N.L., Eichholz D., Rogers S.G. and Fraley R.T. (1985). A simple and general method for transferring genes into plants. *Science* 227, 1229-1231.
- 10 Huang H., Tudor M., Su T., Hu Y. and Ma H. (1996). DNA binding properties of two Arabidopsis MADS domain proteins: Binding consensus and dimer formation. *The Plant Cell* 8, 81-94.
- 15 Jefferson R.A., Kavanagh T.A. and Bevan M. (1987). GUS fusion: J-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* 6, 3901-3907.
- 20 Laemmli U.K. (1970). Cleavage of structural proteins during the assembly of the head bacteriophage T4. *nature* 227, 680-685.
- 25 Lane B.G., Dunwell J.M., Ray J.A., Schmitt M.R. and Cumings A.C. (1993). Germin, a protein marker of early plant development, is an oxalate oxidase. *The Journal of Biological Chemistry* 268 (17), 12239-12242.
- 30 Mariani C., Gossele V., De Beuckeleer M., De Block M., Goldberg R.B., De Greef W. and Leemans J. (1992). a chimaeric ribonuclease-inhibitor gene restores fertility to male sterile plants. *Nature* 357, 384-387.
- 35 Martini M., Schmid A. and Hess D. (1990). Antibiotics, and amino acids in nectar of *Rhododendron* and *Piptanthus* species from Nepal. *Bot. Acta* 103, 343-348.

- 38 -

- Mizukami Y., Huang H., Tudor M. and Ma H. (1995). Functional domains of the floral regulator AGAMOUS: Characterisation of the DNA binding domain and analysis of dominant negative mutations. *The Plant Cell* 8, 831-845.
- 5 Tsuchimoto S., van der Krol A.R. and Chua N.-H, 1993. Ectopic expression of pMADS3 in transgenic petunia phenocopies the petunia *blind* mutant. *Plant Cell* 5, 843-853.
- 10 Verwoerd T.C., Dekker, B.M.M. and Hoekema A. (1989). A small-scale procedure for the rapid isolation of plant RNAs. *Nucl. Acids Res.* 17, 2362.
- 15 Zer H. and Fahn A. (1992). Floral nectaries of *Rosmarinus officinalis* L. Structure, Ultrastructure and Nectar Secretion. *Annals of Botany* 70, 391-397.

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## Sequences:

SEQ ID NO:1 amino acid sequence NEC1

1 MAQLRADDLS FIFGLLGIV SFMVFLAPVP TFYKIYKRKS SEGYQAIPYM  
5  
51 VALFSAGLLL YYAYLRKNAY LIVSINGFGC AIELTYISLF LFYAPRFSKI  
101 FTGWLMLLEL GALGMVMPIT YLLAEGSHRV MIVGWICAAI NVAVFAAPLS  
10 151 IMRQVIKTKS VEFMPFTLSL FLTLCATMWF FYGFFKKDFY IAFPNILGFL  
201 FGIVQMLLYF VYKDSKRIDD EKSDPVREAT KSKEGVEIII NIEDDNSDNA  
251 LQSMKDFSR LRFSK

-40-

SEQ ID No: 2 amino acid sequence of FBP15

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Met Gly Arg Gly Lys Ile Glu Ile Lys Arg Ile Glu Asn Thr Thr Asn
 1          5          10          15
Arg Gln Val Thr Phe Cys Lys Arg Arg Asn Gly Leu Leu Lys Lys Ala
          20          25          30
Tyr Glu Leu Ser Val Leu Cys Asp Ala Glu Val Ala Leu Ile Val Phe
          35          40          45
Ser Ser Arg Gly Arg Leu Tyr Glu Tyr Ala Asn Asn Ser Val Lys Ala
          50          55          60
Thr Ile Asp Arg Tyr Lys Lys Ala Ser Ser Asp Ser Ser Asn Thr Gly
          65          70          75          80
Ser Thr Ser Glu Ala Asn Thr Gln Phe Tyr Gln Gln Glu Ala Ala Lys
          85          90          95
Leu Arg Val Gln Ile Gly Asn Leu Gln Asn Ser Asn Arg Asn Met Leu
          100          105          110
Gly Glu Ser Leu Ser Ser Leu Thr Ala Lys Asp Leu Lys Gly Leu Glu
          115          120          125
Thr Lys Leu Glu Lys Gly Ile Ser Arg Ile Arg Ser Lys Lys Asn Glu
          130          135          140
Leu Leu Phe Ala Glu Ile Glu Tyr Met Arg Lys Arg Glu Ile Asp Leu
          145          150          155          160
His Asn Asn Asn Gln Met Leu Arg Ala Lys Ile Ala Glu Ser Glu Arg
          165          170          175
Asn Val Asn Met Met Gly Gly Glu Phe Glu Leu Met Gln Ser His Pro
          180          185          190
Tyr Asp Pro Arg Asp Phe Phe Gln Val Asn Gly Leu Gln His Asn His
          195          200          205
Gln Tyr Pro Arg Gln Asp Asn Met Ala Leu Gln Leu Val
          210          215          220

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SEQ ID NO:3 amino acid sequence CVSP

5 MFLPILFTISLLFSSSHA

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SEQ ID NO:4 Nucleotide sequence NEC1

5 1 TCGAGCGGCC GCCCGGGCAG GTATTCAACA AGAGTATTCA CCACTTGAAC  
51 TCAAAAGGGG CTTCACTAAA AAAAAATCAT GGCGCAATTA CGTGCTGATG  
101 ACTTGTCTTT CATATTGGC CTTCTTGGTA ATATTGTATC ATTCATGGTC  
10 151 TTCCTAGCAC CCGTGCCAAC ATTTTACAAA ATATATAAAA GGAAATCATC  
201 AGAAGGATAT CAAGCAATAC CATATATGGT AGCACTGTTC AGCGCCGGAC  
15 251 TATTGCTATA TTATGCTTAT CTCAGGAAGA ATGCCTATCT TATCGTCAGC  
301 ATTAATGGCT TTGGATGTGC CATTGAATTA ACATATATCT CTCTGTTTCT  
351 CTTTTACGCG CCCAGAAAGT CTAAGATTTT CACAGGGTGG CTGATGCTCT  
20 401 TAGAATTGGG AGCCCTAGGA ATGGTGATGC CAATTACTTA TTTATTAGCA  
451 GAAGGCTCAC ATAGAGTGAT GATAGTGGGA TGGATTTGTG CAGCTATCAA  
25 501 TGTGCTGTC TTTGCTGCTC CTTTAAGCAT CATGAGGCAA GTAATAAAAA  
551 CAAAGAGTGT AGAGTTCATG CCCTTCACTT TATCTTTGTT CCTCACTCTC  
601 TGTGCCACTA TGTGGTTTTT CTATGGGTTT TTCAAGAAGG ACTTTTACAT  
30 651 TCGGTTTCCA AATATACTGG GCTTTCTATT CGGAATCGTT CAAATGCTAT  
701 TATATTTTGT TTACAAGGAT TCAAAAGAGAA TAGATGATGA AAAATCTGAT  
35 751 CCTGTTCGAG AAGCTACAAA ATCAAAAGAA GGTGTAGAAA TCATTATCAA  
801 CATTGAAGAT GATAATTCTG ATAACGCATT GCAGTCCATG GAGAAGGATT  
851 TTTCCAGACT GCGGACATCA AAATAAGCAA GAAGATGATC AAAAAATGAC  
40 901 AAAGCTAAGG AGTTTGAAGT AAGGCAAGGA ACTTGACACT GAATATCTAA  
951 GCTAATTAGC AAGACTTTAG CAGCTTGTA TATTTAGTGT TTGTGAGGTG  
45 1001 TTACCTTATA ATTAGCTTGT AGCATAGCCT TCCCACTAAT AATTCTGCTT  
1051 AGCGAATCTT ATATATGGGA AATACTTACA CTAGTATGCA TCTTCTATAT  
1101 ACATGTTTGG CACTTGACTA TACATAGAAA AATTAACAAG CATTTCTCAC  
50 1151 CTCAATTTGT CACTTACTTA TAAGTAGCTG AATAATATAA TGCAATTTTC  
1201 ACCCC

55

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SEQ ID NO:5 Nucleotide sequence FBP15

1 TCTGAATACAAGCTGTGTGTGTAGAGAGATTTTCATAAAGACAGCAAACAT  
5 51 CCCTTCTTTTTGTTCTGTTTTAAAGTTCCCTTCTTCAACCAGCTCTTTT  
101 CCTCATCAGGGTAAGTTGCAAATAAAGGGGATGTTCCAGAATCAAGAAGA  
151 GAAGATGTCAGACTCGCCTCAGAGGAAGATGGGAAGAGGAAAGATTGAGA  
10 201 TTAAGAGGATTGAAAATACAACAAATCGTCAAGTCACTTTCTGTAAGAGA  
251 AGAAATGGGTGCTTAAAAAAGCTTATGAACTTTCTGTTCTTTGTGATGC  
15 301 TGAAGTTGCTCTCATCGTTTTCTCAAGCCGTGGCCGCCTCTATGAATATG  
351 CTAACAACAGTGTGAAGGCAACAATTGATAGATATAAGAAAGCATCCTCA  
401 GATTCTCTCAACACTGGATCTACTTCTGAAGCTAACACTCAGTTTTATCA  
20 451 ACAAGAAGCTGCCAAACTCCGAGTTCAGATTGGTAACTTACAGAACTCAA  
501 ACAGGAACATGCTAGGCGAGTCTCTAAGTTCTCTGACTGCAAAAGATCTG  
25 551 AAAGGCCTGGAGACCAAACTTGAGAAAGGAATTAGTAGAATTAGGTCCAA  
601 AAAGAATGAACTCCTGTTTGCTGAGATTGAGTATATGCGAAAAAGGGAAA  
651 TTGATTTGCACAACAACAATCAGATGCTTCGGGCAAAGATAGCTGAGAGT  
30 701 GAAAGAAATGTGAACATGATGGGAGGAGAATTTGAGCTGATGCAATCTCA  
751 TCCGTACGATCCAAGAGACTTCTTCCAAGTGAACGGCTTACAGCATAATC  
35 801 ATCAATATCCACGCCAAGACAACATGGCTCTTCAATTAGTATAAGTTTAT  
851 AATAAAATGCATGGTTTGAAGCACTCTGATTGTGGTGGATTTGGATTATG  
901 TATAAGGGAGTGCAGGCCATTTGCCAATTATTGAAAGGTACTCAAACAGG  
40 951 AAGTTGAAGAAGTTCATCATCTCTCTCATCTATATGTCTTAACAAAAGTC  
1001 TTAGCTTATGGACTCTAAAACAAAGACTTAATTTAACATATAAATATAAT  
45 1051 TGTGTAATGCTGTTGTATTGTATGGTATGTATCCAAAAACATTAATAACC  
1101 TATCTTTTTCTTCAAATTATGTCTCCTTTGATACAACTACTAACATATT  
50 1151 TTCTTAT

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DESC

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SEQ ID NO:6 Nucleotide sequence CVSP

ATGTTTCTTCCAATTCTCTTCACCATTTCCTCCTCTTCTCCTCCTCCCATGCT



- 45 -

SEQ ID NO:7 Nucleotide sequence NEC1 promoter

1 CCTAGGAGAAATCAAGCCTACTCTTAAGATGGATGACTCACTTGCCCCGA  
5 51 TGGTAAGGTGAAGGATCTGTTGATTAGAGTTGGGAAGTTCATGTTCTCTG  
101 CTGATTTTATTATTCTAGACTATGAAGAGGACCAAGAAGCTCCAATAATT  
151 TTGGGAAGAGCATTCTTAATCACATCGATGGCAATTATTGACATGGAAGT  
10 201 TGGGGAGATGACTGTGAGAGCGCATGGAGAAAAGGTTACTTTCAAGGTTT  
251 ATAATAAAAAGGATCATATGGCTAAGTTTGAAGAGTGTTCTTTGATAGAA  
15 301 TGTGTCAGACGAGAACATGAAAGTAAACCGAAAGAGGTGTTTGAGCGGAA  
351 TGTAGAACAAAGTGACCACGGCACAATAATTGACAAGTTGAAGGAAAATT  
401 CACCTAAAGGAAGGAAGAAGACAAAAGTTCGTTCGTAACAAGAGGAGACGT  
20 451 AAATGCTGGAAGTGAGCTTAAAGGTGTTGTCGTACTACGACGTTAACTAA  
501 GCGCTTGTCGGGAGGCAACCCTAGCTTTGTATGTAAATGTAAAAGTAAA  
25 551 AAATATATATATAGAAAAAGGAAAATACAAAAGAGTCGTGCCGCGACGT  
601 TAAATCAAGCGCTTGTTGGAAGGCAACCCAATTTTTATTGTTTTAGTTGT  
651 TTTACTTATTTAGTATTACGTAGTTTCTTGTTGTTTTTGTAGGGCTCGGG  
30 701 ACTTTCGGAAGGTGAGGTAATTTCAAGGCATCGCGGTGTGTATTGCAGCG  
751 AGGTAAGTGTAAGAGTTGAGTTGGAAGCGTTTGCCAAGTGTTGCACCGT  
35 801 GAGAGGCTTCAACCTGTTGCGACACGTGAAAAATTAAGAGCCAGATCTG  
851 CTACATTAGCACTGAAGCATCGCTTGCCAATAGCTTGGAATGGAAGCAA  
901 GAATTCAAACCAAATCAGAAACGCCACAAGAGATGTGTTCGCACACTGCA  
40 951 AAGCTTTGTGCAAACTAGTGAACGCAGAAATAGAAATGCTACAGCCCATG  
1001 CGTCGCTTGGCTTATGGCAGGCAGCAAAAATTCAGCAGCAAAACAGAAAC  
45 1051 GCTGCGAGAAACGCGTCGCATACGCCATAGCTTTGTGTCAAACAGAACGT  
1101 CCAGAAATTGAAAAGCTATAAGCCTGCGTCGCTTGGCTCATGGCGTGCAG  
1151 ACTAGAAAAGCTCTAGCAGATGCGTCGCGTATTGTATAGCTTGGTGTGAA  
50 1201 ACAGAAAGTTCGAAACTTGGAAAACGATAACCCAGCGTCGCCTCTTCAAC  
1251 CGCGTCCAGGTAAGTTCAAGATTCTTACGGGTTGACCCATTAACCCATTG  
55 1301 ATCGGCTGATTATAAACAATAAAACATCACCTTCAACTATCACATGATTT  
1351 CATAAGTTTGACCTAGGATATTTTATATATATATATATATATACACAC  
1401 ACACACCATTTCCAGCGATCTTACCTCATTTTTATTCAAACCATTTTTCT  
60

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1451 GCTTCAAAGTTTAAATTATTAATATGATAAGTCATCCATAGTCAAACAA  
1501 GATTTTCTATACTATTTTGTCCCTTGTAATTTTAAAAAAAAAATGAGCGA  
5 1551 TGGTAAGATAAACATTGTTTGCAAGTGTACAATTTTAGTATATGCAAACC  
1601 AACGCTTCTTCTTCCAACATCACCTAAAACATCATTTATGGCGGGC  
1651 GGACTAGACGTAGCCAAATATAAAAACGCAATGGCCATTCAGTTTCATGTC  
10 1701 ATTTTATATCCTTCATCCAATAATATTACTCAAATTGATGTACAGTTT  
1751 GGTCTCTGATGTGCACTTTACTATACGTAATACGGAATTTACATTATAAT  
15 1801 TAAAGAGAACTGTTCCACTAAATTTTAATGATTTAATTAATTTAACTCGG  
1851 TTAATTGTATTATTATTATTGCTGTATTGTGTTGTCATTTGAATTTGGCA  
1901 CCGCAGATTTTGTATGCAATTAACCCTCATATATCTTTGGCCAAATAA  
20 1951 AGAAAAAGTCTGCATATTTCTTGCCAAACATTTATCATACTTTACCGAAT  
2001 TCTTGTTTTTGTGTTCTCTGTTGTTGTTCTCCACTATAAATAACATTTGC  
25 2051 AGTGAGTAAAGTTTCTTCAGGTCTCTTTGTAGATTCAACAAGAGTATTC  
2101 AGCACTTGAAC TCAAAGGGGCTTCACTAAAAAAATCATG

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SEQ ID NO:8 Nucleotide sequence *FBP15* promoter

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Claims

1. An isolated DNA sequence from the promoter region upstream of a nectary-specific expressed sequence, which nectary-specific expressed sequence encodes a protein comprising the amino acid sequence given in SEQ ID NO: 1, or  
5 a homologous protein.
2. An isolated DNA sequence according to claim 1, wherein the nectary-specific expressed sequence has:  
a) a nucleotide sequence given in SEQ ID NO:4, or  
10 b) a nucleotide sequence obtainable by hybridisation with the nucleotide sequence of (a) or with a fragment of (a).
3. An isolated DNA sequence according to claim 1 or 2, obtained from a plant of *Petunia hybrida*, the sequence  
15 consisting essentially of the sequence given in SEQ ID NO:7, or a functional fragment thereof having promoter activity.
4. An isolated DNA sequence from the promoter region upstream of a nectary-specific expressed sequence, which  
20 nectary-specific expressed sequence encodes a protein comprising the amino acid sequence given in SEQ ID NO:2, or a homologous protein.
5. An isolated DNA sequence according to claim 4, wherein  
25 the nectary-specific expressed sequence has:  
a) a nucleotide sequence given in SEQ ID NO:5, or  
b) a nucleotide sequence obtainable by hybridisation with the nucleotide sequence of (a) or with a fragment of (a).
- 30 6. An isolated DNA sequence according to claim 4 or 5, obtained from a plant of *Petunia hybrida*, the sequence consisting essentially of the sequence given in SEQ ID NO:8 or a functional fragment thereof having promoter activity.

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7. An isolated DNA sequence encoding a protein comprising the amino acid sequence given in SEQ ID NO:1, or a homologous protein, the expression of the DNA sequence being predominantly confined to the nectaries of a plant.

5

8. An isolated DNA sequence according to claim 7 having:

- a) a nucleotide sequence given in SEQ ID NO:4, or
- b) a nucleotide sequence obtainable by hybridisation with the nucleotide sequence of (a) or with a fragment of (a).

10

9. An isolated DNA sequence that results from the sequence shown in SEQ ID NO:4 by insertion, deletion or substitution of one or more nucleotides, including naturally occurring variations or variations introduced by targeted mutagenesis or recombination.

15

10. An isolated DNA sequence according to claim 7 having a nucleotide sequence given in SEQ ID NO:4, said sequence being produced by current DNA synthesis techniques.

20

11. An isolated DNA sequence encoding a protein comprising the amino acid sequence given in SEQ ID NO:2, or a homologous protein, the expression of the DNA sequence being predominantly confined to the nectaries of a plant.

25

12. An isolated DNA sequence according to claim 11, having:

- a) a nucleotide sequence given in SEQ ID NO:5, or
- b) a nucleotide sequence obtainable by hybridisation with

30

the nucleotide sequence of (a) or with a fragment of (a).

13. An isolated DNA sequence that results from the sequence shown in SEQ ID NO:5 by insertion, deletion or substitution of one or more nucleotides, including naturally occurring variations or variations introduced by targeted mutagenesis or recombination.

35

- 50 -

14. An isolated DNA sequence according to claim 11 having a nucleotide sequence given in SEQ ID NO:5, said sequence being produced by current DNA synthesis techniques.

5 15. Any sequence that encodes a nectary-specific MADS box gene.

10 16. An isolated DNA sequence comprising the coding region for a signal peptide, wherein the information contained in the DNA sequence permits, upon translational fusion with a DNA sequence encoding a protein that is expressed in nectaries, targeting of the protein to nectar.

15 17. An isolated DNA sequence according to claim 16, comprising the nucleotide sequence given in SEQ ID NO: 6 obtained from a plant of *Calluna vulgaris*, or a nucleotide sequence obtainable by hybridisation with the nucleotide sequence given in SEQ ID NO:6.

20 18. A recombinant double-stranded DNA molecule comprising an expression cassette comprising the following constituents:  
i) a promoter functional in plants,  
25 ii) a DNA sequence coding for a protein as defined in any of claims 7 to 15 which is fused to the promoter sequence in sense or antisense orientation, and optionally  
iii) a signal sequence functional in plants for the transcription termination and polyadenylation of an RNA  
30 molecule.

19. A recombinant double-stranded DNA molecule comprising an expression cassette comprising the following constituents:  
i) a promoter functional in nectaries of plants,  
35 ii) a DNA sequence coding for a protein which is fused to the promoter sequence in sense or antisense orientation, and optionally

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iii) a signal sequence functional in plants for the transcription termination and polyadenylation of an RNA molecule.

5 20. A recombinant double-stranded DNA molecule comprising an expression cassette comprising the following constituents:

- i) a promoter functional in nectaries of plants,
- ii) a DNA sequence encoding a protein which is fused to the promoter,
- 10 iii) a DNA sequence encoding a signal peptide that targets the recombinant protein to nectar, which is translationally fused to the DNA sequence encoding the recombinant protein, and optionally
- 15 iv) a signal sequence functional in plants for the transcription termination and polyadenylation of an RNA molecule.

21. A recombinant double-stranded DNA molecule according to claim 19 or 20 wherein the promoter is as defined in any of  
20 claims 1-6.

22. A recombinant double-stranded DNA molecule according to claim 20 or 21 wherein the DNA sequence encoding a signal peptide is as defined in claim 16 or 17.

25 23. A process for producing a transgenic plant exhibiting excretion of a recombinant protein in its nectar, comprising:

- 30 i) introducing in a plant cell a recombinant double-stranded DNA-molecule as defined in any of claims 20 to 22, wherein the recombinant protein is excreted in nectar
- ii) regenerating plants from the transgenic cell, and
- iii) selecting transgenic plants.

35 24. A process for producing a transgenic plant exhibiting a modified nectar composition, comprising:

- i) introducing in a plant cell a recombinant double-stranded DNA-molecule as defined in any of claims 19 to 22,



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wherein the recombinant protein interferes with metabolic pathways in the nectaries,

- ii) regenerating plants from the transgenic cell, and
- iii) selecting transgenic plants.

5

25. A process for producing a transgenic plant exhibiting a modified nectar secretion, comprising:

- i) introducing in a plant cell a recombinant double-stranded DNA-molecule as defined in any of claims 19 to 22, wherein the recombinant protein interferes with sink strength of nectaries

10

- ii) regenerating plants from the transgenic cell, and
- iii) selecting transgenic plants.

15

26. A process for producing a transgenic plant exhibiting a modified nectary development, comprising:

- i) introducing in a plant cell a recombinant double-stranded DNA-molecule as defined in claims 19 or 22, wherein the recombinant protein interferes with the development of nectaries

20

- ii) regenerating plants from the transgenic cell, and
- iii) selecting transgenic plants.

25

27. A process for producing honey from modified nectar of transgenic plants, comprising:

- i) producing a transgenic plant by introducing in a plant cell a recombinant double-stranded DNA molecule as defined in any of claims 19 to 22, regenerating plants from the transgenic cell, and selecting modified plants exhibiting the excretion of nectar with a modified composition,

30

- ii) allowing insects, preferably bees, to collect nectar from the transgenic plants and to process the nectar into honey.

35

28. A process for producing a recombinant gene product from honey, comprising:

- 53 -

- 5 i) producing a transgenic plant by introducing in a plant cell a recombinant double-stranded DNA molecule as defined in any of claims 20 to 22, regenerating plants from the transgenic cell, and selecting modified plants exhibiting excretion of the recombinant gene product in nectar,
- ii) allowing insects, preferably bees, to collect nectar from the transgenic plants and to process the nectar into honey, and
- 10 iii) isolating and purifying the gene product from the honey.

29. A process for producing a metabolite from honey, comprising:

- 15 i) producing a plant that excretes this metabolite in nectar and which plant has been produced by current breeding and selection methods,
- ii) allowing insects, preferably bees, to collect nectar from the selected plants and to process the nectar into
- 20 honey, and
- iii) isolating and purifying the metabolite from the honey.

30. Micro organisms containing DNA sequences according to one or more of claims 1 to 17.

25

31. Micro organisms containing recombinant DNA molecules according to any of claims 18 to 22.

30 32. A plant cell or plant cell culture transformed with one or more DNA sequences according to claims 1 to 17.

33. A plant cell or plant cell culture transformed with recombinant DNA molecules according to any of 18 to 22.

35

34. A plant consisting essentially of the plant cells of claims 32 or 33.

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35. A transgenic plant obtained by the process of any of claims 23 to 26.

5 35. Seeds, tissue culture, plant parts or progeny plants derived from a transgenic plant according to claim 35.

36. Honey obtained from nectar from transgenic plants, which nectar has a modified composition.

10 37. Honey obtained from nectar from transgenic plants, which nectar comprises a recombinant gene product.

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1 TGATCCTGTT CGAGAAGCTA CAAAATCAAA AGAAGGTGTA GAAATCATT A  
51 TCAACATTGA AGATGATAAT TCTGATAACG CATTGCAGTC CATGGAGAAG prat 119 ←  
101 GATTTTTCCA GACTGCGGAC ATCAAAATAA GCAAGAAGAT GATCAAAAAA  
151 TGACAAAGCT AAGGAGTTTG AAGTAAGGCA AGGAACTTGA CACTGAATAT  
201 CTAAGCTAAT TAGCAAGACT TTAGCAGCTT GTAATATTTA GTGTTTGTGA  
251 GGTGTTACCT TATAATTAGC TTGTAGCATA GCCTTCCCAC TAATAATTCT prat 122 ←  
301 GCTTAGCGAA TCTTATATAT GGGAAATACT TACACTAGTA TGCATCTTCT  
351 ATATACATGT TTGGCACTTG ACTATACATA GAAAAATTAA CAAGCATTTC  
401 TCACCTCAAT TTGTCACTTA CTTATAAGTA GCTGAATAAT ATAATGCAAT  
451 TTTCACCCC

FIG.2

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1 TCGAGCGGCC GCCCGGGCAG GTATTCAACA AGAGTATTCA CCACTTGAAC  
51 TCAAAAGGGG CTTCACTAAA AAAAAATCAT GGCGCAATTA CGTGCTGATG prat 129 →  
101 ACTTGCTCTT CATATTTGGC CTTCTTGGTA ATATTGTATC ATTCATGGTC  
151 TTCCTAGCAC CCGTGCCAAC ATTTTACAAA ATATATAAAA GGAAATCATC  
201 AGAAGGATAT CAAGCAATAC CATATATGGT AGCACTGTTC AGCGCCGGAC  
251 TATTGCTATA TTATGCTTAT CTCAGGAAGA ATGCCTATCT TATCGTCAGC  
301 ATTAATGGCT TTGGATGTGC CATTGAATTA ACATATATCT CTCTGTTTCT  
351 CTTTTACGCG CCCAGAAAGT CTAAGATTTT CACAGGGTGG CTGATGCTCT  
401 TAGAATTGGG AGCCCTAGGA ATGGTGATGC CAATTACTTA TTTATTAGCA  
451 GAAGGCTCAC ATAGAGTGAT GATAGTGGGA TGGATTTGTG CAGCTATCAA  
501 TGTGCTGTC TTTGCTGCTC CTTTAAGCAT CATGAGGCAA GTAATAAAAA  
551 CAAAGAGTGT AGAGTTCATG CCCTTCACTT TATCTTTGTT CCTCACTCTC  
601 TGTGCCACTA TGTGGTTTTT CTATGGGTTT TTCAAGAAGG ACTTTTACAT  
651 TCGTTTTCCA AATATACTGG GCTTCTATT CGGAATCGTT CAAATGCTAT  
701 TATATTTTGT TTACAAGGAT TCAAAGAGAA TAGATGATGA AAAATCTGAT  
751 CCTGTTTCGAG AAGCTACAAA ATCAAAAGAA GGTGTAGAAA TCATTATCAA  
801 CATTGAAGAT GATAATTCTG ATAACGCATT GCAGTCCATG GAGAAGG

FIG.3

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1 TCGAGCGGCC GCGCGGCAG GTATTCAACA AGAGTATTCA CCACTTGAAC  
51 TCAAAAGGGG CTTCACTAAA AAAAAATCAT GCGCAATTA CGTGCTGATG  
101 ACTTGTCTTT CATATTTGGC CTTCTTGGA ATATTGTATC ATTCATGGTC  
151 TTCCTAGCAC CCGTGCCAAC ATTTTACAAA ATATATAAAA GGAAATCATC  
201 AGAAGGATAT CAAGCAATAC CATATATGGT AGCACTGTTC AGCGCCGGAC  
251 TATTGCTATA TTATGCTTAT CTCAGGAAGA ATGCCTATCT TATCGTCAGC  
301 ATTAATGGCT TTGGATGTGC CATTGAATTA ACATATATCT CTCTGTTTCT  
351 CTTTTACGCG CCCAGAAAGT CTAAGATTTT CACAGGGTGG CTGATGCTCT  
401 TAGAATTGGG AGCCCTAGGA ATGGTGATGC CAATTACTTA TTTATTAGCA  
451 GAAGGCTCAC ATAGAGTGAT GATAGTGGGA TGGATTTGTG CAGCTATCAA  
501 TGTGCTGTC TTTGCTGCTC CTTTAAGCAT CATGAGGCAA GTAATAAAAA  
551 CAAAGAGTGT AGAGTTCATG CCCTTCACTT TATCTTTGTT CCTCACTCTC  
601 TGTGCCACTA TGTGGTTTTT CTATGGGTTT TTCAAGAAGG ACTTTTACAT  
651 TGC GTTTCCA AATATACTGG GCTTTCTATT CGGAATCGTT CAAATGCTAT  
701 TATATTTTGT TTACAAGGAT TCAAAGAGAA TAGATGATGA AAAATCTGAT  
751 CCTGTTTCGAG AAGCTACAAA ATCAAAAGAA GGTGTAGAAA TCATTATCAA  
801 CATTGAAGAT GATAATTCTG ATAACGCATT GCAGTCCATG GAGAAGGATT  
851 TTTCCAGACT GCGGACATCA AAATAAGCAA GAAGATGATC AAAAAATGAC  
901 AAAGCTAAGG AGTTTGAAGT AAGGCAAGGA ACTTGACACT GAATATCTAA  
951 GCTAATTAGC AAGACTTTAG CAGCTTGTA TATTTAGTGT TTGTGAGGTG  
1001 TTACCTTATA ATTAGCTTGT AGCATAGCCT TCCCACTAAT AATTCTGCTT  
1051 AGCGAATCTT ATATATGGGA AATACTTACA CTAGTATGCA TCTTCTATAT  
1101 ACATGTTTGG CACTTGACTA TACATAGAAA AATTAACAAG CATTTCTCAC  
1151 CTCAATTTGT CACTTACTTA TAAGTAGCTG AATAATATAA TGCAATTTTC  
1201 ACCCC

FIG.4



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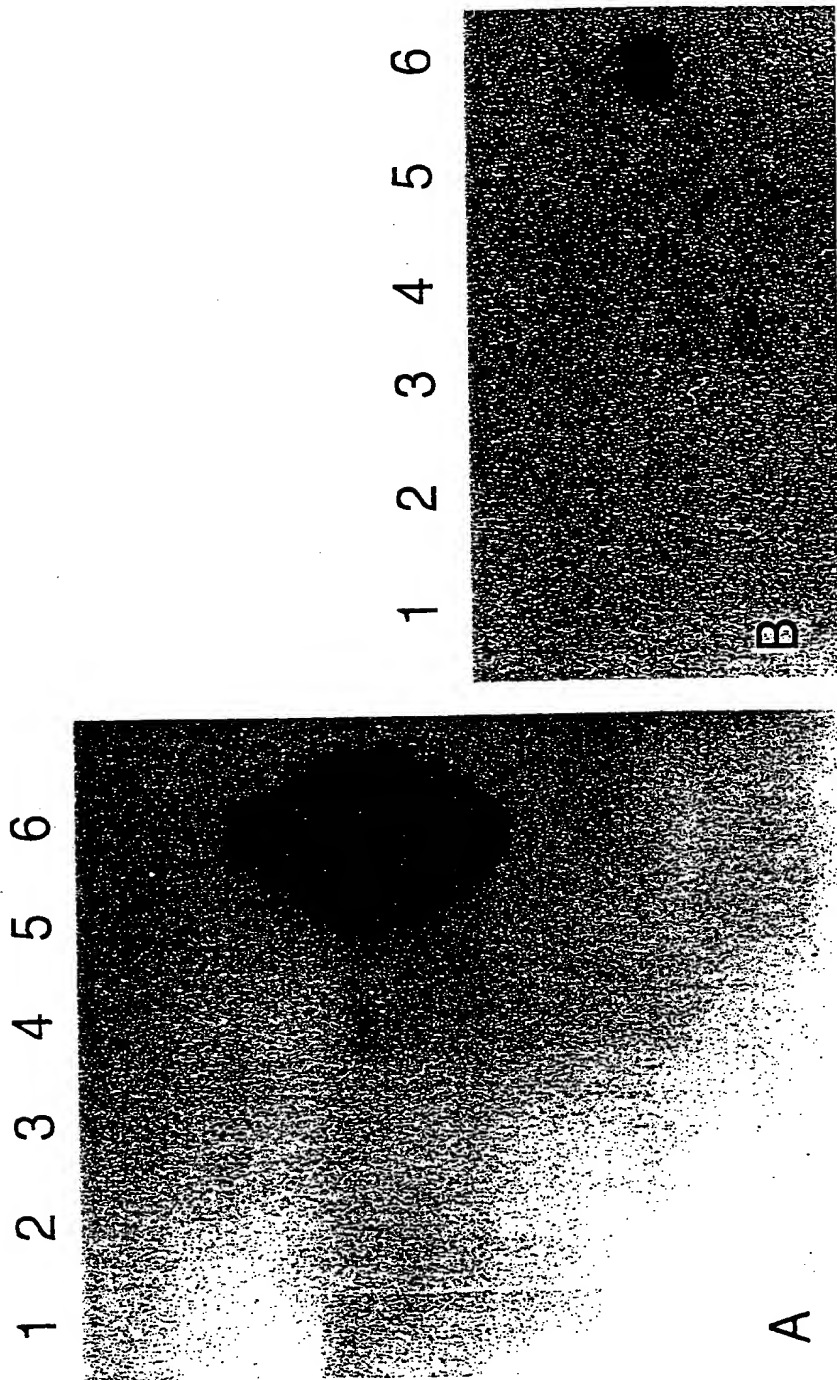


FIG. 5

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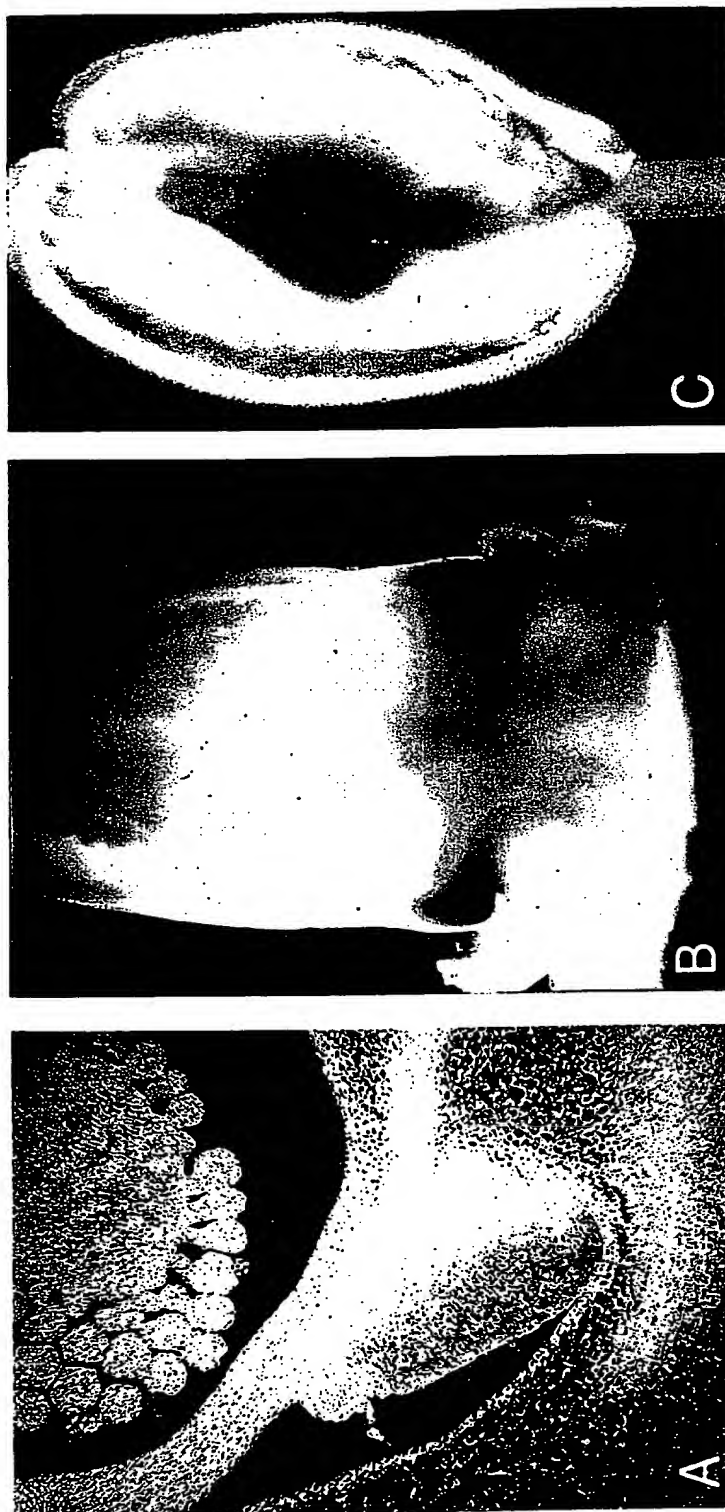


FIG. 6

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1 CCTAGGAGAAATCAAGCCTACTCTTAAGATGGATGACTCACTTGCCCCGA  
51 TGGTAAGGTGAAGGATCTGTTGATTAGAGTTGGGAAGTTCATGTTCTCTG  
101 CTGATTTTATTATTCTAGACTATGAAGAGGACCAAGAAGCTCCAATAATT  
151 TTGGGAAGAGCATTCTTAATCACATCGATGGCAATTATTGACATGGAAGT  
201 TGGGGAGATGACTGTGAGAGCGCATGGAGAAAAGGTTACTTTCAAGGTTT  
251 ATAATAAAAAGGATCATATGGCTAAGTTTGAAGAGTGTTCTTTGATAGAA  
301 TGTGTCAGACGAGAACATGAAAGTAAACCGAAAGAGGTGTTTGAGCGGAA  
351 TGTAGAACAAAGTGACCACGGCACAATAATTGACAAGTTGAAGGAAAATT  
401 CACCTAAAGGAAGGAAGAAGACAAAAGTTCGTCGTAACAAGAGGAGACGT  
451 AAATGCTGGAAGTGAGCTTAAAGGTGTTGTCGTACTACGACGTAACTAA  
501 GGCGCTTGTCGGGAGGCAACCCTAGCTTTGTATGTAAATGTAAAAGTAAA  
551 AAATATATATATAGAAAAAGGAAAATACAAAAGAGTCGTGCCGCGACGT  
601 TAAATCAAGCGCTTGTTGGAAGGCAACCCAATTTTTATTGTTTTAGTTGT  
651 TTTACTTATTTAGTATTACGTAGTTTCTTGTTGTTTTGTAGGGCTCGGG  
701 ACTTTCGGAAGGTGAGGTAATTTCAAGGCATCGCGGTGTGTATTGCAGCG  
751 AGGTAAGTGTAAAGAGTTGAGTTGGAAGCGTTTGGCCAAGTGTTGCACCGT  
801 GAGAGGCTTTCAACCTGTTGCGACACGTGAAAAATTAAGAGCCAGATCTG  
851 CTACATTAGCACTGAAGCATCGCTTGGCCAATAGCTTGGAATGGAAGCAA  
901 GAATTCAAACCAAATCAGAAACGCCACAAGAGATGTGTGCGCAGACTGCA  
951 AAGCTTTGTGCAAACTAGTGAACGCAGAAATAGAAATGCTACAGCCCATG  
1001 CGTCGCTTGGCTTATGGCAGGCAGCAAAAATTCAGCAGCAAAACAGAAAC  
1051 GCTGCGAGAAACGCGTCGCATACGCCATAGCTTTGTGTCAAACAGAACGT  
1101 CCAGAAATTGAAAAGCTATAAGCCTGCGTCGCTTGGCTCATGGCGTGCAG  
1151 ACTAGAAAAGCTCTAGCAGATGCGTCGCGTATTGTATAGCTTGGTGTGAA  
1201 ACAGAAAGTTCGAAACTTGGAACGATAACCCAGCGTCGCCTCTTCAAC  
1251 CGCGTCCAGGTAAGTTCAAGATTCTTACGGGTTGACCCATTAACCCATTG  
1301 ATCGGCTGATTATAAACAATAAAACATCACCTTCAACTATCACATGATTT  
1351 CATAAGTTTGACCTAGGATATTTTATATATATATATATATATATACACAC

FIG.7

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1401 ACACACCATTTCCAGCGATCTTACCTCATTTTTATTCAAACCATTTTTCT  
1451 GCTTCAAAAGTTTAAATTATTAATATGATAAGTCATCCATAGTCAAACAA  
1501 GATTTTCTATACTATTTTGTCCCTTGTAATTTTAAAAAAAAAATGAGCGA  
1551 TGGTAAGATAAACATTGTTTGCAAGGTGACAATTTTAGTATATGCAAACC  
1601 AACGCTTCTTCTTCCAACATACACCTAAACTACATCATTTATGGCGGGC  
1651 GGACTAGACGTAGCCAAATATAAAAACGCAATGGCCATTGAGTTCATGTC  
1701 ATTTTATATCCTTCATCCAATAATATTACTCAAAATTGATGTACAGTTT  
1751 GGTCTCTGATGTGCACTTTACTATACGTAATACGGAATTTACATTATAAT  
1801 TAAAGAGAACTGTTCCACTAAATTTAATGATTTAATTAATTTAACTCGG  
1851 TTACTTGTATTATTATTATTGCTGTATTTGTTTGTCATTTGAATTTGGCA  
1901 CCGCAGATTTTGTATGCAATTAACCCTCATATATCTTTGGCCAAATAA  
1951 AGAAAAAGTCTGCATATTTCTTGCCAAACATTTATCATACTTTACCGAAT  
2001 TCTTGTTTTTTGTTTCTCTGTTGTTGTTCTCCACTATAAATAACATTTGC  
2051 AGTGAGTAAAGTTTCTTCAGGTCTCTTTTGTAGATTCAACAAGAGTATTC  
2101 AGCACTTGAACCTCAAAGGGGCTTCACTAAAAAAAATCATG

FIG.7 (CONTIN.)

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## PBNEP1

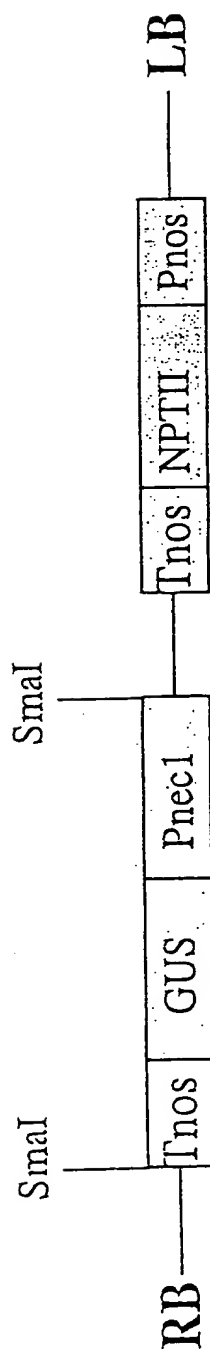


FIG. 8

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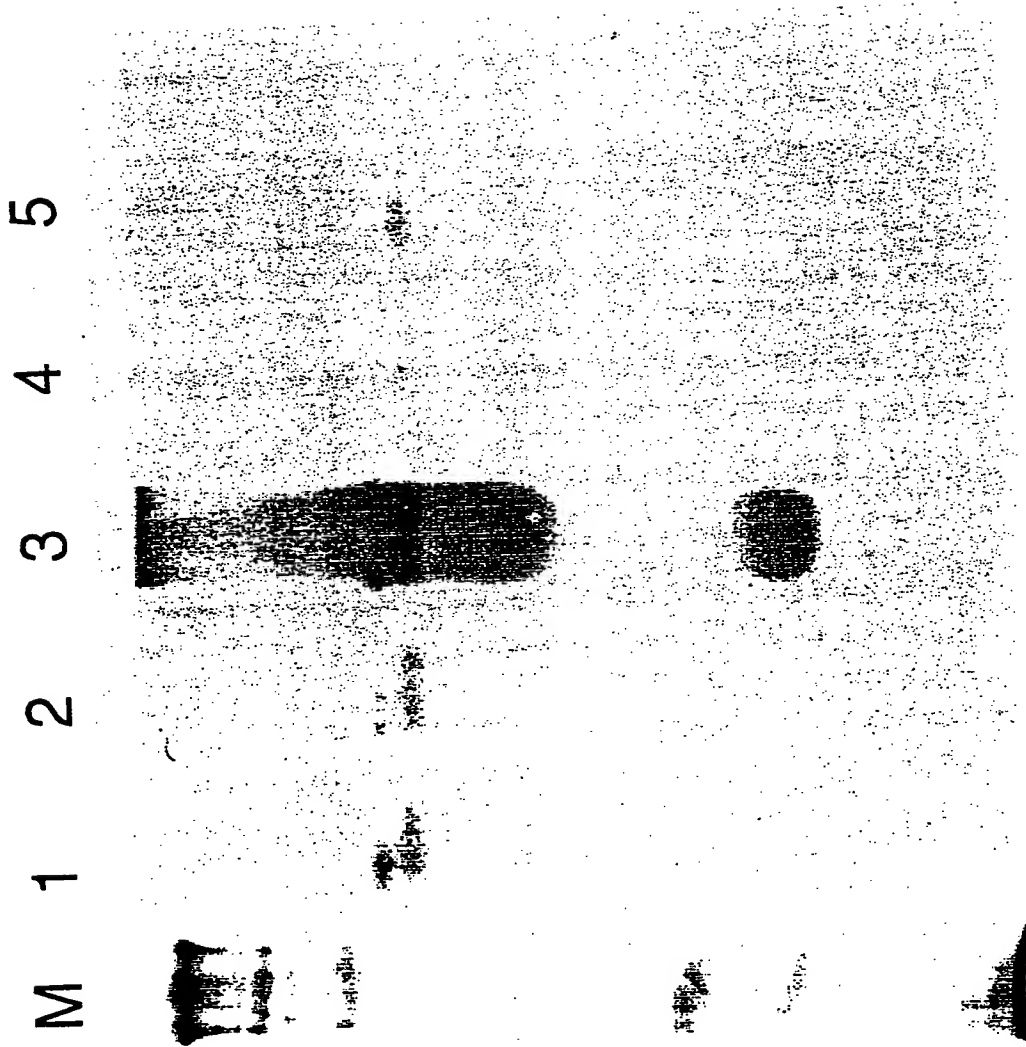


FIG.9

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# SDS page of honey and nectar

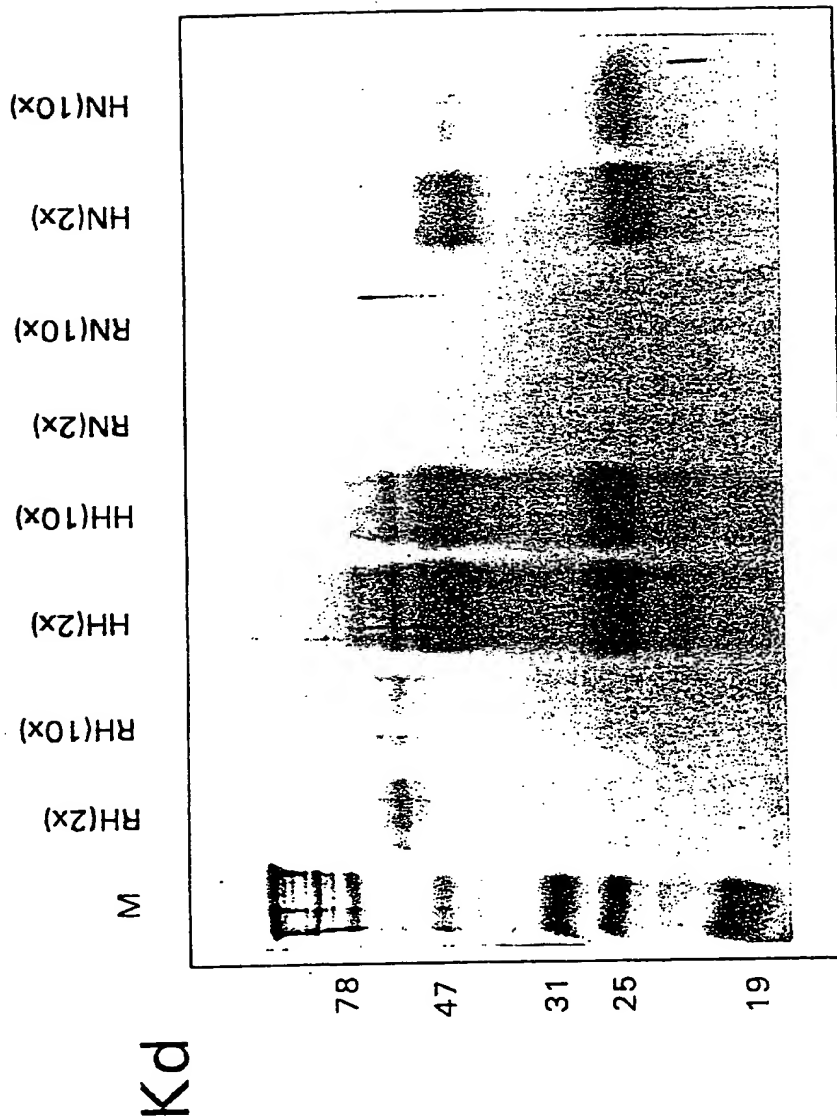


FIG.10

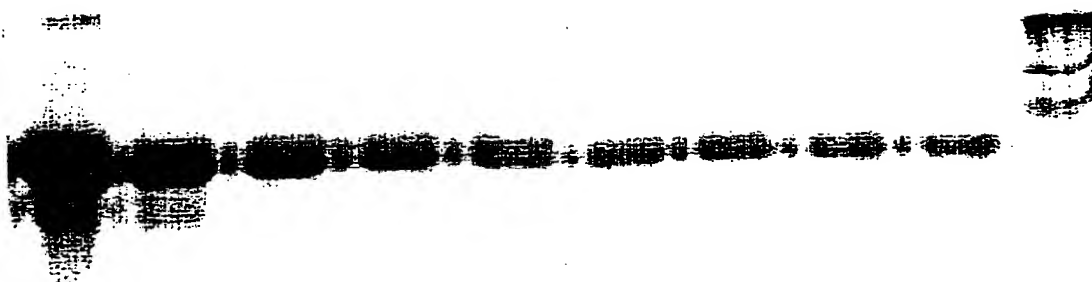
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1 2 3 4 5 6 7 8 9



A

1 2 3 4 5 6 7 8 9 M



B

FIG. 11



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====17-APR-1998=====PC/GENE====

\*\*\*\*\*  
 \* ALIGNMENT OF TWO PROTEIN SEQUENCES. \*  
 \*\*\*\*\*

The two sequences to be aligned are:

PCVH29.

Total number of residues: 60.

GER1.

Total number of residues: 211.

Comparison matrix : Structure-genetic matrix.

Open gap cost : 7

Unit gap cost : 1

The character to show that two aligned residues are identical is '|'

```

PCVH29  - MKMFLPILFTISLLFSSSHASVLDFCVADPSLPDGPAGYSCKEPAKVTVD -50
          |||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||
GER1     - MKMRIQIFFILSLFSSISFASVQDFCVADPKGPNPSGYCKNPDQVTEN -50
                                     -60
PCVH29  - DFVFHGLGTA
          |||  ||  ||  ||  ||
GER1     - DFAFSGLGKAGNTSNVIKAAVTPAFAPAFAGLNGLDVSLARLDLAGGGVI -100
                                     -150
GER1     - PLHTHPGASEVLVVIQGTICAGFISSANKVYLKTLRGDSMVFPQGLLHF
                                     -200
GER1     - QLNSGKGPALAFVAFGSSSPGLQILPFALFANDLPSELVEATTFLSDEEV
GER1     - KKLKGVLGGTN -211
  
```

Identity : 36 ( 60%)

Number of gaps inserted in PCVH29: 0

Number of gaps inserted in GER1: 0

====17-APR-1998=====PC/GENE====

FIG.12

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→ prat 176

Ser - Val - Leu - Asp - Phe - Cys - Val - Ala - Asp - Pro - Ser - Leu - Pro  
 TCA GT\* CT\* GAC TTC TGC GT\* GC\* GAC CC\* TC\* CT\* CC\*  
 TCC TTA GAT TTT TGT GAT AGC TTA TTG  
 TCG TCT AGC AGT

Asp - Gly - Pro - Ala - Gly - Tyr - Ser - Cys - Lys - Glu - Pro - Ala - Lys - Val  
 GAC GG\* CC\* GC\* GG\* TAC TC\* TGC AAA GAA CC\* GC\* TGC GT\*  
 TAT AGC TGT AAG GAG TGT

← prat 177

Thr - Val - Asp - Asp - Phe - Val - Phe - His - Gly - Leu - Gly - Thr - Ala  
 AC\* GT\* GAC GAC TTC GT\* TTC CAC GG\* CT\* GG\* AC\* GC\*  
 GAT GAT TTT CAT TTA TTG

A

Ser - Val - Leu - Asp - Phe - Cys - Val - Ala - Asp - Pro - Ser - Leu - Pro  
 TCA GT\* CT\* GAT TTC TGT GTG GCT GAT CCA TCC TTG CCC  
 TCC TTA TTG  
 TCG TCT AGC AGT

prat 206 ↔ prat 204  
 prat 207 ↔ prat 205

Asp - Gly - Pro - Ala - Gly - Tyr - Ser - Cys - Lys - Glu - Pro - Ser - Lys - Val  
 GAT GGC CCT GCA GGC TAC TCC TGC AAG GAG CCC TCT AAA GTC

Thr - Val - Asp - Asp - Phe - Val - Phe - His - Gly - Leu - Gly - Thr - Ala  
 ACC GTA GAC GAT TTC GT\* TTC CAC GG\* CT\* GG\* AC\* GC\*  
 TTA TTG

B

FIG. 13

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MAR5H6	CGCCCGGCTGGTAAACAAAGTACGTGTATACCTATTGTTTCCTTAAA	50
MAR5R8	CGCCCGGCTGGTAAACAAAGTACGTGTATACCTATTGTTTCCTTAAA	50
MAR5R6	CGCCCGGCTGGTAAACAAAGTACGTGTATACCTATTGTTTCCTTAAA	50
MAR5H8	CGCCCGGCTGGTAAACAAAGTACGTGTATACCTATTGTTTCCTTAAA	50
	*****	
MAR5H6	AAAGCAACTGTCAAAATTCGACCAAAACGAGTATATAAGTATCACTTTCC	100
MAR5R8	AAAGCAACTGTCAAAATTCGACCAAAACGAGTATATAAGTATCACTTTCC	100
MAR5R6	AAAGCAACTGTCAAAATTCGACCAAAACGAGTATATAAGTATCACTTTCC	100
MAR5H8	AA-GCAACTGTCAAAATTCGACCAAAACGAGTATATAAGTATCACTTTCC	99
	** *****	
MAR5H6	CCCTATTGGACAAACGAACTCTAAGAGGGCAATCAGACACACAGCCAT	150
MAR5R8	CCCTATTGGACAAACGAACTCTAAGAGGGCAATCAGACACACAGCCAT	150
MAR5R6	CCCTATTGGACAAACGAACTCTAAGAGGGCAATCAGACACACAGCCAT	150
MAR5H8	CCCTATTAAACACACGAACTCTAAGAGGGCAATCAGACACACAGCCAT	149
	*****	
MAR5H6	TGCACCTGTGAAGATGTTTCTTCCAATTCCTTCACCATTTCCCTCCTCT	200
MAR5R8	TGCACCTGTGAAGATGTTTCTTCCAATTCCTTCACCATTTCCCTCCTCT	200
MAR5R6	TGCACCTGTGAAGATGTTTCTTCCAATTCCTTCACCATTTCCCTCCTCT	200
MAR5H8	TGCACCTATGAAGATGTTTCTTCCAATTCCTTCACCATTTCCCTCCTCT	199
	*****	
	signal sequence ← mature protein	
MAR5H6	TCTCTCTCTCCATGCTTCAGTGTGGACTTCTGGTAGCAGACCCATCC	250
MAR5R8	TCTCTCTCTCCATGCTTCAGTGTGGACTTCTGGTAGCAGACCCATCC	250
MAR5R6	TCTCTCTCTCCATGCTTCAGTGTGGACTTCTGGTAGCAGACCCATCC	250
MAR5H8	TCTCTCTCTCCATGCTTCAGTGTGGACTTCTGGTAGCAGACCCATCC	249
	*****	

FIG.14

14-12-1998

EP98204215.2

DRAW

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atgttcctccaattcctttcactattttctctctctctctctctctctcatgcttctgttcttgatttcg  
acaaggaagcttaagaaaagtcataaacagagaaaaagagaaagagctacgagacaagaactaaagcctag

FIG. 15

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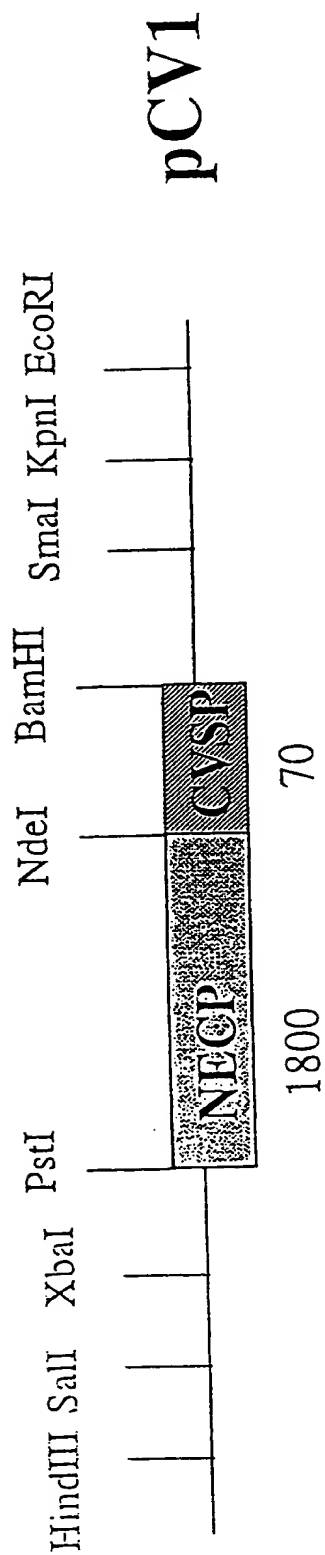


FIG.16

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pCV2

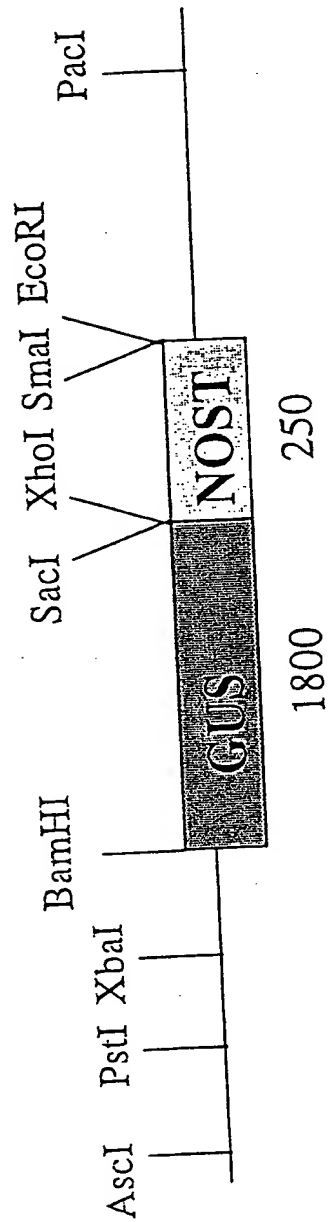


FIG.17

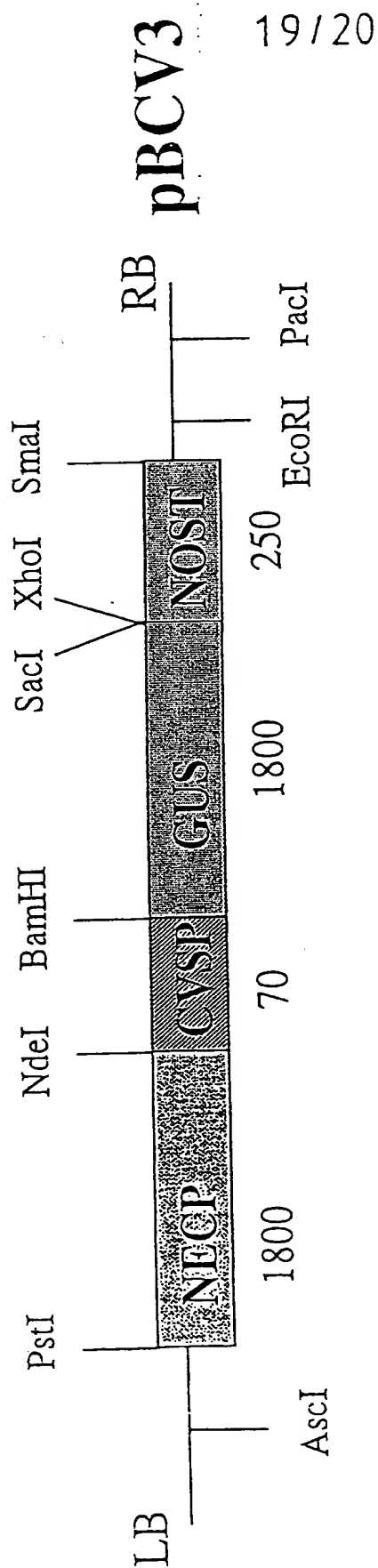


FIG. 18

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1 TCTGAATACAAGCTGTGTGTGTAGAGAGATTTCATAAAGACAGCAAACAT  
51 CCCTTCTTTTGTCTGTTTTAAAAGTTCCCTTCTTCAACCAGCTCTTTT  
101 CCTCATCAGGGTAAGTTGCAAATAAAGGGGATGTTCCAGAATCAAGAAGA  
151 GAAGATGTCAGACTCGCCTCAGAGGAAGATGGAAGAGGAAAGATTGAGA  
201 TTAAGAGGATTGAAAATACAACAAATCGTCAAGTCACTTTCTGTAAAGAGA  
251 AGAAATGGGTGCTTAAAAAGCTTATGAACCTTTCTGTTCTTTGTGATGC  
301 TGAAGTTGCTCTCATCGTTTCTCAAGCCGTGGCCGCCTCTATGAATATG  
351 CTAACAACAGTGTGAAGGCAACAATTGATAGATATAAGAAAGCATCCTCA  
401 GATTCCCTCCAACACTGGATCTACTTCTGAAGCTAACACTCAGTTTTATCA  
451 ACAAGAAGCTGCCAAACTCCGAGTTCAGATTGGTAACTTACAGAACTCAA  
501 ACAGGAACATGCTAGGCGAGTCTCTAAGTTCTCTGACTGCAAAAGATCTG  
551 AAAGGCCTGGAGACCAAACCTTGAGAAAGGAATTAGTAGAATTAGGTCCAA  
601 AAAGAATGAACCTCCTGTTTGCTGAGATTGAGTATATGCGAAAAAGGAAA  
651 TTGATTTGCACAACAACAATCAGATGCTTCGGGCAAAGATAGCTGAGAGT  
701 GAAAGAAATGTGAACATGATGGGAGGAGAATTTGAGCTGATGCAATCTCA  
751 TCCGTACGATCCAAGAGACTTCTTCCAAGTGAACGGCTTACAGCATAATC  
801 ATCAATATCCACGCCAAGACAACATGGCTCTTCAATTAGTTAAGTTTAT  
851 AATAAAATGCATGGTTTGAAGCACTCTGATTGTGGTGGATTGATTATG  
901 TATAAGGGAGTGCAGGCCATTGCCAATTATTGAAAGGTACTCAAACAGG  
951 AAGTTGAAGAAGTTCATCATCTCTCTCATCTATATGTCTTAACAAAAGTC  
1001 TTAGCTTATGGACTCTAAAACAAGACTTAATTTAACATATAAATATAAT  
1051 TGTGTAATGCTGTTGTATTGTATGGTATGTATCCAAAAACATTAATAACC  
1101 TATCTTTTCTTCAAATTATGTCTCCTTTGATACAACTACTAACATATT  
1151 TTCTTAT

MADS-box

K-box

FIG. 19



EPO - DG 1

- 55 -

14 12. 1998

## Abstract

Process to collect metabolites from modified nectar by insects

The invention relates to a recombinant double-stranded DNA molecule comprising an expression cassette comprising the following constituents:

- i) a promoter functional in nectaries of plants,
- 5 ii) a DNA sequence encoding a protein which is fused to the promoter,
- iii) a DNA sequence encoding a signal peptide that targets the recombinant protein to nectar, which is translationally fused to the DNA sequence encoding the recombinant protein, and optionally
- 10 iv) a signal sequence functional in plants for the transcription termination and polyadenylation of an RNA molecule.

The invention further relates to a process for producing a recombinant gene product from honey, comprising:

- 15 i) producing a transgenic plant by introducing in a plant cell a recombinant double-stranded DNA molecule, regenerating plants from the transgenic cell, and selecting modified plants exhibiting excretion of the recombinant gene product in nectar,
- 20 ii) allowing insects, preferably bees, to collect nectar from the transgenic plants and to process the nectar into honey, and
- iii) isolating and purifying the gene product from the
- 25 honey.

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